Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the detection of HIV-1 drug resistance mutations

Dissertation submitted as a part of fulfilment of the rules and regulations for the M.D. (Branch- IV Microbiology) examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in May 2018

CERTIFICATE

This is to certify that the dissertation titled, "**Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the detection of HIV-1 drug resistance mutations**" is the bonafide work of Dr. Priyanka Sabu in partial fulfilment of the rules and regulations for the M.D. (Branch- IV Microbiology) examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in May 2018.

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DECLARATION

I hereby declare that this MD dissertation titled "Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the detection of HIV-1 drug resistance mutations" is the bonafide work done by me under the guidance of Dr. Rajesh Kannangai, Professor and Head, Dept. of Clinical Virology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

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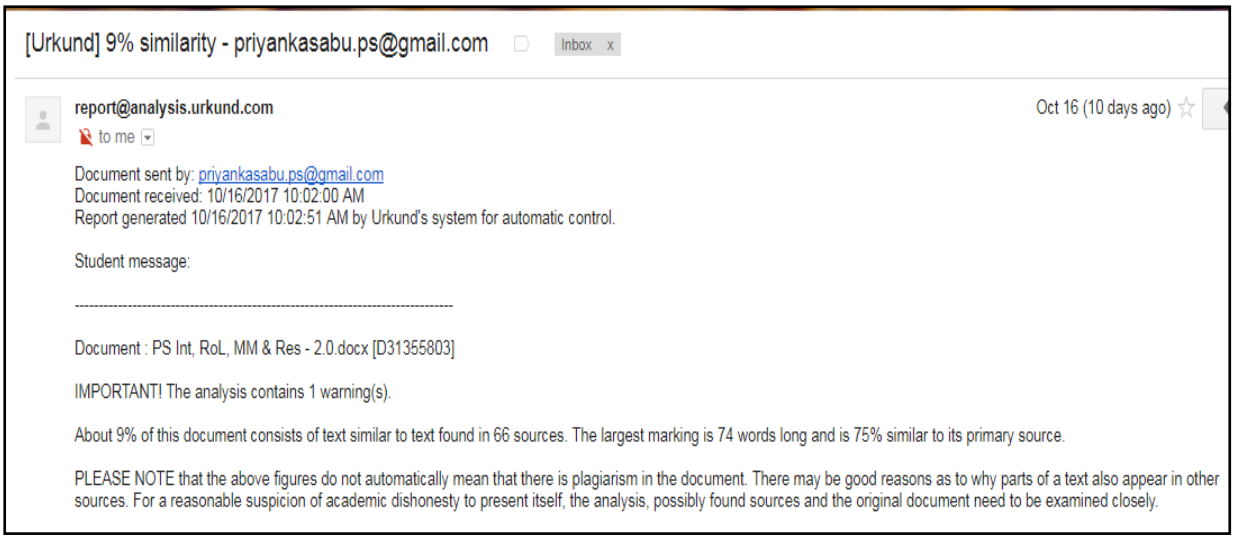

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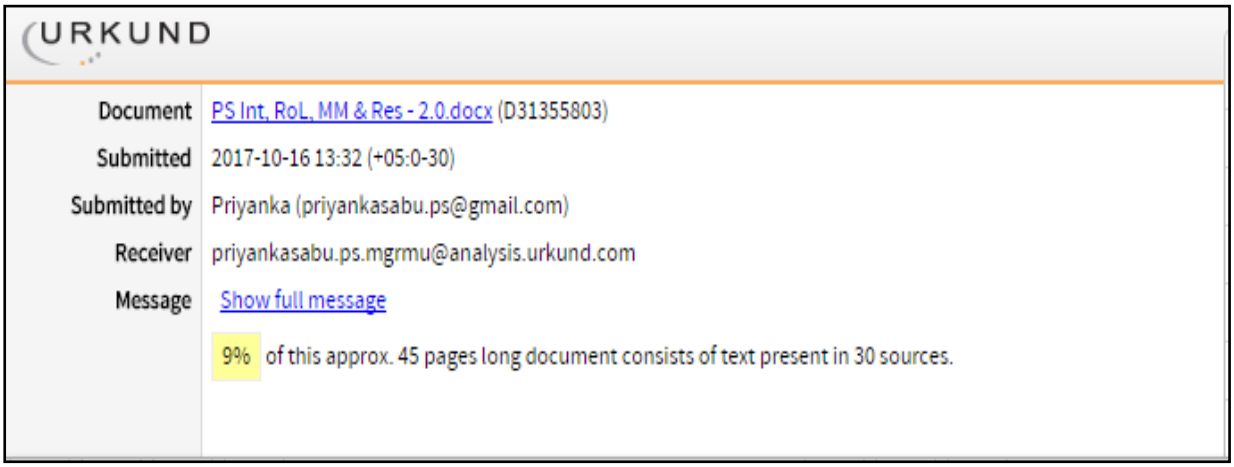

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Annexure

1. Introduction

Human immunodeficiency virus (HIV) infection can lead to a clinical disease spectrum extending from being asymptomatic to advanced immunological incompetency as a consequence of quantitative and qualitative inadequacy of T lymphocytes resulting in a stage known as Acquired Immunodeficiency Syndrome (AIDS), where the affected persons are left susceptible to a host of life threatening opportunistic infections and malignancies which account for most of the symptoms seen in HIV infected individuals (1). Since it was first observed in 1981, HIV infection has turned into an explosive pandemic which has left no region of the world untouched, causing significant morbidity and mortality (2).

HIV is a diploid single-stranded RNA virus belonging to the family *Retroviridae* and genus *Lentivirus.* There are two types of the virus – HIV-1 and HIV-2. The former, identified separately in Paris and United States, is responsible for approximately 99% of all human infections globally (3). The latter, isolated in 1986 in West Africa (4), is associated with lower levels of viremia and transmission rates and also has a slower progression of the disease when compared to HIV-1 (5).

HIV is transmitted through unprotected sexual intercourse, parenteral route by transfusion or through sharing of needles and from a mother to her child during pregnancy, during childbirth and breastfeeding (6).

Though there is no cure for HIV infection, appropriate treatment with antiretroviral drugs can control the virus and suppress its replication, turning a fatal disease into a lifelong chronic infection. The number of AIDS related deaths (ARD) and associated morbidity have been remarkably reducing over the years. This fall corresponds with the worldwide increase in access of people living with HIV to antiretroviral therapy (ART) from 7.7 million in 2010 to 19.5 million by 2016 (7). A similar trend is seen in India, following the rapid extension of

easy and free access to ART (8) with approximately 1.04 million individuals receiving ART in India, having a coverage of about 49% among people living with HIV as of December 2016 (9).

However the advent of drug resistant mutations has put the long-term management of HIV/AIDS at risk. Thus making resistance testing among ART-experienced individuals who are failing their current regimen, crucial. Also the use of resistance testing in the choice of the initial therapy has proved to result in a greater decrease in viral load and is cost-effective. Additionally it is important in the monitoring of individuals on treatment and for surveillance of drug resistance in the community to help select appropriate treatment regimens (10) (11). Following the trends in disease evolution has shown that HIV-1 drug resistance testing is a decisive part in the management of HIV infection.

Of the two methods available to test for HIV resistance, genotypic assays are the gold standard and plasma is the most appropriate clinical sample (12), since it is known to have HIV-1 RNA at higher and more stable levels than serum and whole blood (13). Also, it should be collected while the patient continues to be on the failing ART regimen to sustain the selective pressure on the viral populations (14). Plasma samples must be separated from blood cells within 6 hours of collection to prevent RNA degradation and is to be stored at -70^{0}C until the time of testing (12). These genotypic assays are available only in select laboratories in India. The lack of adequate facilities and equipment, and the effort involved with maintaining the cold-chain of plasma during transportation and storage at the tropical temperature, leaves the resource-limited settings incapable of managing HIV-1 infected individuals satisfactorily (15).

Alternate, practical and reliable means to obtain, store and transport blood samples are essential to develop cost effective assays in such settings. Dried blood spot (DBS) is being studied as an alternative specimen to plasma. The ease of collection of whole blood samples

onto a DBS card, its storage at room temperature and transport to reference laboratories at ambient temperature has made DBS an appealing sample for HIV-1 drug resistance testing (15).

Many studies have reported the successful genotyping of HIV-1 from DBS and some have shown a high genotypic concordance with plasma genotypes. During the past few years DBS has started to be used widely for HIV-1 drug resistance testing world over and an increased number of reports from resource-limited areas have indicated DBS as the preferred specimen for transmitted HIV-1 drug resistance surveillance where collection of plasma is not feasible (16).

However, from India there is only minimal information available on drug resistance genotyping assays for the detection of HIV-1 drug resistance mutations using DBS samples stored at ambient temperature.

Furthermore, apart from the mutations in HIV RNA, additional mutations may be present in the proviral DNA which was integrated into the host cell genome. From the plasma sample, only the mutations in viral RNA can be detected but from DBS samples, mutations in both viral RNA and proviral DNA can be detected. And the origin of the additionally detected mutations can be confirmed by drug resistance testing of peripheral blood mononuclear cells (PBMC).

These reasons lead to the need to undertake a study to test for drug resistance mutations from corresponding plasma, DBS cards and PBMC samples and to evaluate the efficacy of DBS for the detection of HIV-1 drug resistance and the concordance in the mutations observed in the three sample types.

2. Hypothesis and Objectives

Hypothesis

Dried blood spot (DBS) is as efficient as plasma sample for the detection of HIV-1 drug resistance mutations.

Objectives

1) To sequence HIV-1 *pol* gene from plasma, DBS and PBMC to assess drug resistance mutations in the reverse transcriptase and protease regions in individuals showing treatment failure.
2) To compare the frequency of HIV-1 drug resistance mutations detected in plasma, DBS and PBMC to confirm the origin of mutations.
3) To evaluate the efficacy of DBS for detection of HIV-1 drug resistance mutations in samples stored at ambient temperature for 10 days.

3. Literature Review

3.1 Discovery

In 1981, Centre for Disease Control (CDC) released an unusual report of five previously healthy young homosexual men who suffered from Pneumocystis carinii pneumonia (PCP) in United States. Their case histories suggested that the cell-mediated immune system was impaired and was probably due to a disease transmitted sexually (17). The disease was recognised as a syndrome and termed 'acquired immunodeficiency syndrome (AIDS)' in 1982. Already AIDS seemed to be a long-lasting illness with an extended duration between exposure, through blood or sexual activity and the state of dramatic immune dysfunction which was plagued with opportunistic infections or malignancies (18).

Human T-cell leukaemia viruses (HTLV) was considered to be the causative agent since the different manifestations of AIDS were unified by a depletion of CD4 T-cells. Apart from the leukaemia and lymphomas, HTLV also caused an AIDS-like wasting syndrome and was transmitted through blood, sexual route and from mother to child, thus justifying the assumption. Independently, the pursuit of a HTLV-like virus in patients with AIDS was started in the National Institute of Health (NIH), Bethesda and the Pasteur Institute, Paris (18). In 1983, Luc Montagnier and his scientists from Pasteur Institute isolated the virus from a homosexual patient with generalised hyperplastic lymphadenopathy and called it Lymphadenopathy associated virus (LAV), a unique human retrovirus (19). In NIH, Robert Gallo who had previously discovered HTLV types I and II also isolated the primary cause of AIDS and named it HTLV type III in the year 1984 (20). Around the same time, another group of scientists from the University of California, San Francisco under the leadership of Dr. Jay Levy identified the virus and called it AIDS-associated retrovirus (ARV) (21).

In 1986, the International Committee on Taxonomy of Viruses stated that the retrovirus which was recognised as the etiologic agent of AIDS was to be renamed as Human immunodeficiency virus (HIV) to remove the multiple names that was in circulation then (22). The first confirmation of HIV infection in India was from female commercial sex workers at a custodial care institution in Tamil Nadu (23).

3.2 Epidemiology

3.2.1 Global scenario

In three and a half decades, HIV has infected more than 70 million people and has led to roughly 35 million deaths worldwide. By the end of the year 2016, there were 36.7 million people living with HIV globally with a probable prevalence ranging between 0.7% and 0.9% in adults aged 15 to 49 years. And mortality due to HIV-related illness for the year 2016 was 1 million people. The disease burden is variable between different regions of the world, the worst affected being sub-Saharan Africa where 1 in every 25 adults is living with HIV which accounts for approximately 70% of people living with HIV worldwide (24).

3.2.2 Indian scenario

In India, the estimated prevalence of HIV in adults between 15 and 49 years of age is 0.26%, according to the India HIV Estimation Report for the year 2015. The highest prevalence of 1.15% is seen in the state of Manipur which is followed by Mizoram with 0.80%, Nagaland with 0.78%, Andhra Pradesh and Telangana with 0.66%, Karnataka having 0.45%, Gujarat with 0.42% and Goa having 0.40%. Also Maharashtra, Chandigarh, Tripura and Tamil Nadu have an adult HIV prevalence rate more than the national prevalence. Whereas Odisha, Bihar, Sikkim, Delhi, Rajasthan and West Bengal have an adult HIV prevalence ranging from 0.21% to 0.25%. The remaining states and union territories have a prevalence below the national average. The national prevalence of HIV among adults has gradually been decreasing from a

peak of 0.38% in 2001-2003 through 0.34% in 2007 and 0.28% in 2012 to 0.26% in 2015 (25).

The number of people living with HIV (PLHIV) was estimated to be 22.26 lakhs in 2007 and 21.17 lakhs in 2015. Andhra Pradesh and Telangana together have the peak number of PLHIV with 3.95 lakhs, which is followed by Maharashtra with 3.01 lakhs and Karnataka having 1.99 lakhs. These states along with Gujarat, Bihar and Uttar Pradesh make up 64.4% of the projected PLHIV in India (8).

The prevalence among high risk groups like female sex workers (2.2%), men who have sex with men (4.3%), transgender (7.5%) and intravenous drug users (9.9%) is assessed by the National Integrated Behavioural and Biological Surveillance (8). However the virus nor the disease is not limited to the high risk groups alone. HIV-1 is transmitted to the general low risk population through a bridging population. They bridge the gap between the high risk group and the general population and include long distance truck drivers and migrant labourers (26) who maybe clients or partners of commercial sex workers (27).

3.3 Structure

HIV is an enveloped, spherical shaped virus particle of 100 nm in size. The lipid bi-layered viral envelope is derived from the host cell membrane and is embedded with major envelope proteins, glycoprotein 120 (gp120) and glycoprotein 41 (gp41) which form the knob-like surface and anchoring transmembrane spikes, respectively. In between the envelope and the core is the matrix which is largely made of Gag protein p17. Its inner core, composed of p24 capsid protein, is cylindrical or conical in shape and contains two identical copies of single stranded positive sense viral RNA closely associated with Gag protein p7 along with the enzymes, reverse transcriptase, integrase and protease which are essential for replication and propagation of the virus (1) as shown in **Figure 1.**

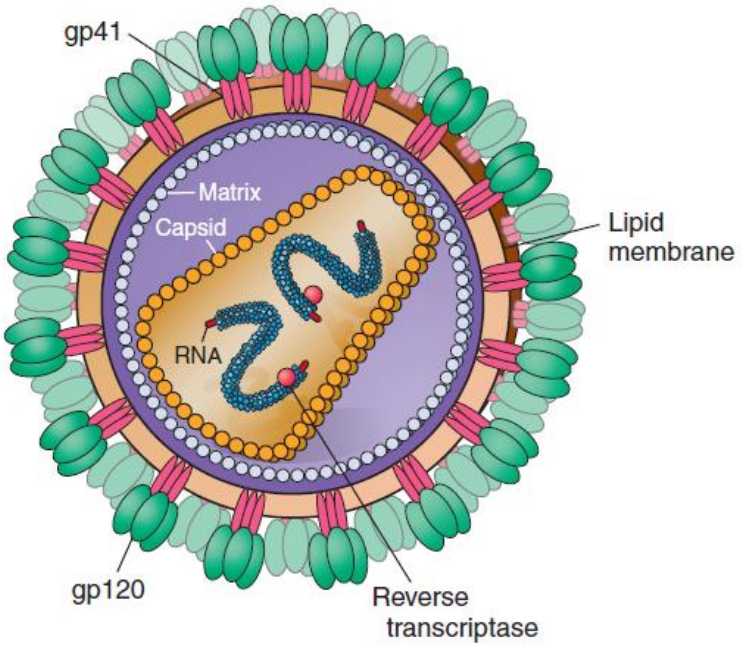


Figure 1: Structure of HIV-1 *(Adapted from Harrison's Principles of Internal Medicine, 19th edition)*

3.4 Genome

HIV-1 genome is 9.7 kbp in length and includes major genes which code for three groups of structural and enzymatic proteins, six genes encoding non-structural regulatory and accessory proteins, which are flanked by the long terminal repeat sequences (LTR) (28) as shown in **Figure 2**.

The three major structural genes are:

- *Gag* gene: Encodes for the proteins forming the viral capsid (p24), nucleocapsid (p7), matrix (p17) and p6.
- *Pol* gene: Codes for the enzymes – protease (p10), reverse transcriptase (p66/51) and integrase (p32).
- *Env* gene: Expresses a large precursor glycoprotein (gp160) which is cleaved into surface protein (gp120) which mediates CD4 and chemokine receptor binding and into transmembrane protein (gp41) which acts as the fusion protein (1) (6).

The six non-structural genes are divided into regulatory genes (*tat*, *rev* and *nef*) that code for regulatory proteins – transcriptional activator (p14), regulator protein (p19) and negative regulator protein (p27) respectively and into accessory genes (*vif*, *vpr* and *vpu*) that code for accessory proteins – viral infectivity factor (p23), viral protein R (p15) and viral protein U (1).

The long terminal repeat sequences present at both ends of the genome contain promoter and enhancer sequences that are required for initiation of transcription (1,6,28).

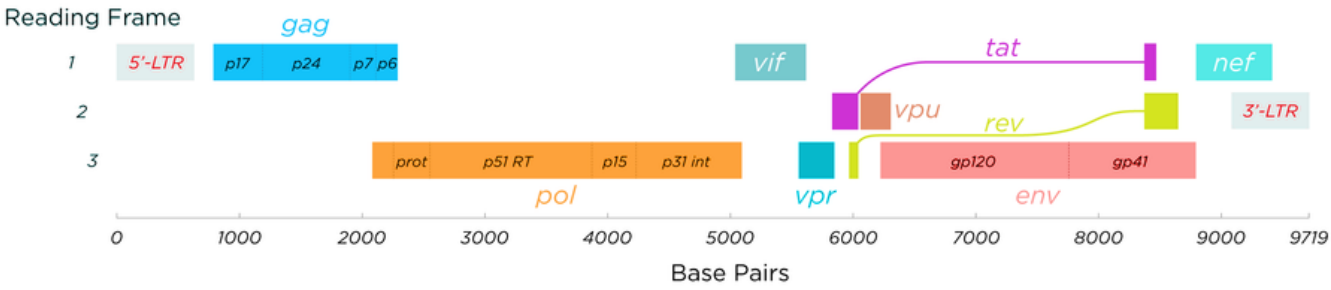


Figure 2: HIV-1 RNA genomic structure *(Adapted from Scientific Illustration, www.scistyle.com)*

3.5 Molecular classification

HIV infection was seen in human beings following zoonotic infections with simian immunodeficiency viruses (SIV) from African primates. HIV-1 was transmitted from chimpanzees (SIVcpz) and HIV-2 from sooty mangabey apes (SIVsm) (29).

The phylogenetic clustering of global HIV-1 viral isolates shows four groups: M (Main/ Major), O (Outlier), N (non-M, non-O) and P (30) which represent four separate cross-species transmission events (31).

HIV-1 group M is responsible for the pandemic of HIV-1 infections, due to its many subtypes and circulating recombinant forms (CRFs). There are nine recognised subtypes of group M, which are A, B, C, D, F, G, H, J and K. Though all subtypes are said to have originated from

central Africa, each has a distinct geographical distribution and risk group association (30,32). Subtype B is commonly seen in western Europe, America and Australia, subtype C predominantly in Africa and most parts of Asia including India, subtype E in Thailand and subtype F in South America (6). Within a subtype, the variation at amino acid level is 8-17% and between subtypes it is 17-35%. Recombination of strains are seen between different HIV-1 groups and also between and within group M subtypes. CRFs are recombinants of different group M subtypes which were sequenced and found in 3 or more epidemiologically unconnected individuals (30,32). There are around 90 existing CRFs presently (33).

Groups N and O have been limited to a small number of people in Cameroon and in Cameroon, Gabon and Equatorial Guinea, respectively where their prevalence is extremely low. Group P has been identified only in 2 individuals from Cameroon (32).

3.6 Genetic diversity

The vast genetic unpredictability and rapid evolution of HIV-1 have contributed significantly to the global spread of the virus. This genetic variability is due to the considerable mutation and recombination rates of the reverse transcriptase enzyme which does not have a proof-reading mechanism and is accompanied by high viral replication rates. Mutations like insertions and deletions are common in HIV-1 genome. Such mechanisms have led to the generation of virus populations that are genetically diverse within each infected person. Viral sequences can vary up to 10% within a single individual (32).

3.7 Replication

The principle target of HIV is the immune system, specifically, activated CD4 T lymphocytes. The virion attaches to the host cell by the interaction of its envelope glycoprotein, gp120 with the CD4 molecule of T helper cells followed by binding to chemokine co-receptors, CCR5

and CXCR4 (31). The virus penetrates the host cell by the fusion of the viral envelope with the host cell membrane, aided by the exposed gp41 molecule. Following fusion, matrix and capsid proteins are digested and the viral enzymes and RNA are released into the host cell cytoplasm. The reverse transcriptase (RT) enzyme utilizes host nucleotides and forms a single-stranded DNA from the viral RNA, which is then transformed to a double-stranded DNA copy (34). This double-stranded DNA is then transported into the nucleus of the infected cell along with the integrase enzyme which inserts the viral DNA into the host cell DNA. In this state, it is called a *provirus* and the infection is permanent hereafter (6). It is able to replicate using the host cell replication machinery. Subsequently, transcription takes place to produce viral RNA and mRNA which is translated into viral proteins and processed to form virion components in the cytoplasm of the host cell. Immature virions are assembled under the organization of *gag* polyproteins at the cell membrane where the envelope and core proteins are located. Maturation of the immature virion can occur either while it separates from the host cell by a process called budding or thereafter. The protease (PR) enzyme cleaves the polyproteins to their functional size, thus generating a complete mature virion that is capable of infecting another cell (34–36).

The life cycle of the retrovirus therefore involves two forms, a DNA provirus and a RNA containing infectious virion.

3.8 Transmission

HIV can be transmitted by an active free virus or a latent virus hidden within infected cells (34). The presence of the virus in blood, semen, cervical and vaginal secretions leads to its transmission (6) through the following three different routes:

- Heterosexual or homosexual sexual contact (vaginal, oral or anal) with an infected partner.
- Parenteral route by transfusion of tainted blood and blood products, organ transplants from infected donors, sharing of needles or syringes with infected individuals or needle stick injuries from contaminated sharps.
- From an infected mother to her child during pregnancy, childbirth or breastfeeding (34).

A vast majority (90%) of the global total of HIV infections occurs through heterosexual contact even though the risk of transmission from one unprotected encounter is as low as 0.1-0.2% (6). The factors that determine the risk of sexual transmission are the plasma HIV-1 RNA viral load of the infected partner (37), the frequency of sexual contact and presence of genital ulcers due to other sexually transmitted infections (31,38) like herpes simplex-2, syphilis or bacterial vaginosis which may increase the risk of transmission 300 times over (34). Pregnancy, receptive anal intercourse and behavioural features like homosexuality and multiple sexual partners are associated with increased risk of sexual transmission whereas male circumcision with reduced risk (31).

The probability of acquiring HIV infection through infected blood products is estimated to be > 90% (1) however, it has dramatically reduced due to the mandatory screening for blood-borne infections prior to transfusions and organ transplantations. Owing to the practise of sharing unsterilized needles, syringes and related paraphernalia has put injection drug users at three times a higher risk of infection than through sexual transmission (34). The risk depends on the duration of injection drug use, the frequency of sharing needles, the number of people with whom they are shared and such practices in a geographic setting with high prevalence of HIV infection (1). Health-care workers are at risk of HIV through occupational

exposure to accidental penetrating needle stick injuries and splashes to conjunctiva, other mucous membranes or non-intact skin with contaminated blood (6).

The transmission from a HIV infected mother to her child can take place during antenatal period, perinatal period or via breastfeeding. The rate of transmission ranges from 15-45% if no interventions are taken, while it is ≤ 1% with effective interventions like ART for infected pregnant and breastfeeding mothers, a short course for the baby and good breastfeeding practices (39,40).

3.9 Pathogenesis

The disease is characterized by severe immunodeficiency due to progressive quantitative and qualitative depletion of CD4+ helper T cells which are the primary targets of HIV. The observed cellular deficiency and dysfunction of CD4+ cells are due to various mechanisms like: direct infection and destruction of the cells and/ or indirectly by immune clearance of infected cells and immune fatigue following aberrant activation (1), as listed in **Table 1**.

Table 1: Mechanisms of CD4 depletion *(Adapted from Harrison's Principles of Internal Medicine, 19th edition)*

Direct mechanisms	**Indirect mechanisms** (41)
• Syncytia formation • Accumulation of unintegrated viral DNA • Alteration of plasma membrane permeability due to viral budding • Interference with cellular RNA processing	• Apoptosis and autoimmunity • Infected cells killed by HIV-specific immune response • Inhibition of T cell production by thymus • Bystander killing of viral antigen-coated cells

HIV infection generally charts the following progression. It has an acute stage of marked viral replication and dissemination, then a chronic asymptomatic phase of continued immune activation and viral replication and finally the advanced stage of AIDS (42).

After virus entry, there is a period of unrestrained virus multiplication in the target cells indicated by non-specific symptoms of a viral illness like fever, fatigue, lymphadenopathy, rash and myalgia, the high viral RNA and p24 antigen in circulation and a transient fall in CD4+ cell counts. The viremia leads to dissemination of the virus to all the lymphoid tissue (1,6,43). Once the infection is established it persists lifelong.

Slowly the immune system responds, both viral RNA and p24 antigen fall to a level where p24 antigen becomes undetectable and viral load gets fixed at a low level called the set-point (6,43). In spite of the vigorous immune response following the primary infection, HIV manages to escape immune-mediated elimination and instead flourishes on immune activation and develops into a chronic persistent infection which may last for approximately 10 years. The inability to clear the infection completely is due to the development of post-integration latency in infected CD4+ cells where the integrated HIV provirus remains latent until further activation (1).

The ratio of infected CD4+ cells and viral RNA level rises as the disease progresses until the individual becomes symptomatic. The persistent assault on the immune system impairs it, leading to the worsening of symptoms as the immunity deteriorates and progression to AIDS. Once the CD4+ cell counts fall below 200 cells/μl, the infected individual is susceptible to a host of life-threatening opportunistic infections which the immune system normally would have been able to prevent and has an increased risk of various malignancies (1,34). The course of events in an untreated HIV-infected person is shown in **Figure 3**.

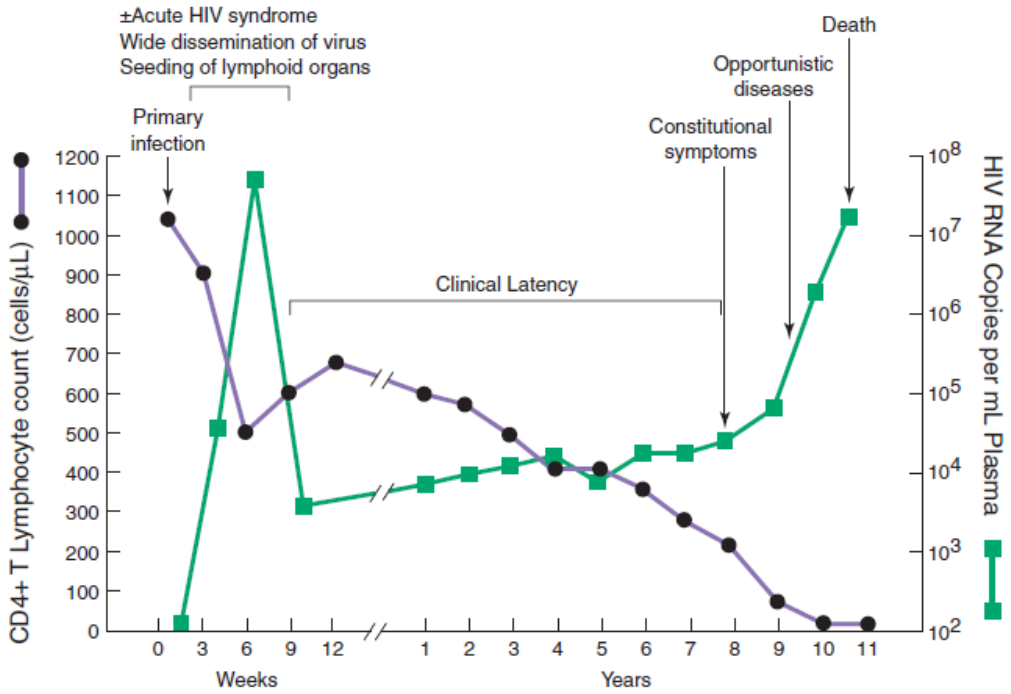


Figure 3: Viral load and CD4+ cell count during the course of disease in an untreated individual. *(Adapted from Harrison's Principles of Internal Medicine, based on an original from Pantaleo et al, N Engl J Med 328:327, 1993)*

3.10 Classification of HIV

HIV disease classification and staging systems are decisive for monitoring HIV infected individuals and their disease progression, thus guiding the clinicians with its management. The two main systems that are being used are: the World Health Organization (WHO) clinical staging system and the Centre for Disease Control (CDC) classification system.

The WHO system does not entirely depend on CD4 cell counts or other diagnostic tests, hence can be easily used in resource limited scenarios (44).

Table 2: WHO Clinical Staging of HIV/ AIDS (45)

Clinical Stage	Manifestations
Stage 1	Asymptomatic
	Persistent generalized lymphadenopathy (PGL)
Stage 2	Moderate unexplained weight loss (<10% of body weight)
	Recurrent upper respiratory tract infections

	Herpes zoster, papular pruritic eruption
	Angular cheilitis, recurrent oral ulceration
	Seborrheic dermatitis, fungal nail infections
Stage 3	Severe unexplained weight loss (>10% of body weight)
	Inexplicable chronic diarrhoea (> 30 days)
	Inexplicable persistent fever (> 30 days)
	Persistent oral candidiasis, oral hairy leucoplakia
	Pulmonary tuberculosis
	Severe systemic bacterial infections
	Acute necrotizing ulcerative oral lesions
	Inexplicable pancytopenia
Stage 4	HIV wasting syndrome
	Recurrent severe bacterial pneumonia, *Pneumocystis* pneumonia
	Extra pulmonary tuberculosis
	Disseminated nontuberculous mycobacterial infection
	Chronic herpes simplex infection, cytomegalovirus infection
	Oesophageal candidiasis,
	Extra pulmonary cryptococcosis, disseminated mycosis
	Chronic cryptosporidiosis, chronic isosporiasis
	Central nervous system toxoplasmosis
	Atypical disseminated leishmaniasis
	Kaposi sarcoma
	Lymphoma (cerebral or B-cell non-Hodgkin)
	Invasive cervical carcinoma
	Progressive multifocal leukoencephalopathy
	Symptomatic HIV-associated nephropathy or encephalopathy

The CDC classification depends on the lowest CD4 cell count that was recorded and on any prior HIV-related illness that was diagnosed. Individuals who fit into categories A3, B3 and C1-C3 are thought to have AIDS.

Table 3: CDC Classification System for HIV (46)

CD4 cell counts (cells/ µl)	Categories		
	A*	**B#**	**C†**
> 500	A1	B1	C1
200 – 499	A2	B2	C2
< 200	A3	B3	C3

* **Category A:** Asymptomatic HIV infection, acute or primary HIV, persistent generalised lymphadenopathy (PGL)

Category B: Symptomatic, not A or C manifestations like oropharyngeal or vulvovaginal candidiasis, herpes zoster, cervical dysplasia, fever or diarrhoea > 1 month.

† **Category C:** AIDS-indicator diseases like pulmonary and disseminated tuberculosis, Pneumocystis *jiroveci* pneumonia, oesophageal candidiasis, extra pulmonary cryptococcosis, CMV, Kaposi's sarcoma, lymphoma, HIV-related wasting syndrome, encephalopathy.

3.11 Diagnosis of HIV infection

Determining the HIV status of an infected person can be done only by laboratory testing. It can be performed by directly detecting the presence of the virus (RNA or DNA provirus) or viral products (p24 antigen), on the other hand, for indirect detection, the immune response (HIV-specific antibodies) to HIV infection can also be measured. The appropriate method to be used for laboratory detection of HIV relies on its natural history and the time since exposure when an individual comes for testing, as depicted in **Figure 4**.

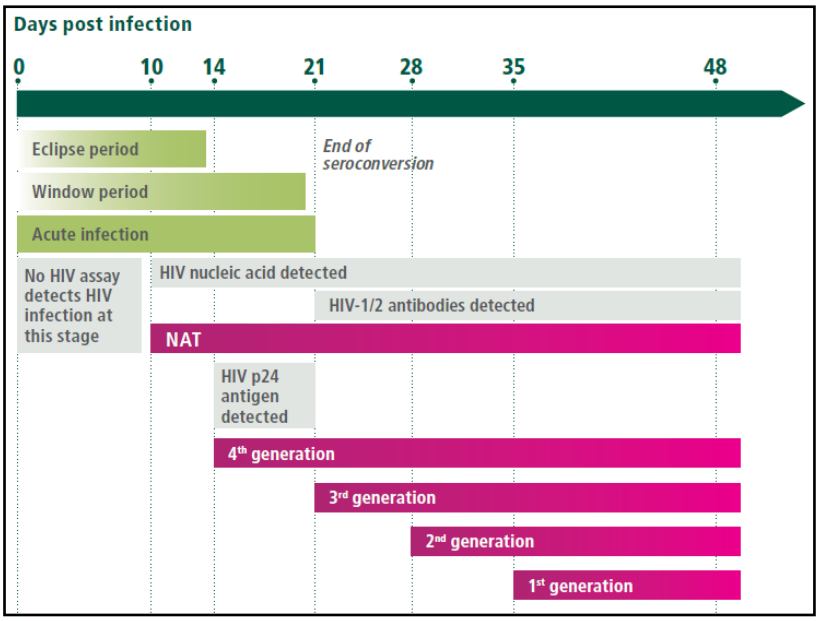


Figure 4: Different HIV detection methods to be used through the course of the disease. *(Adapted from WHO Consolidated Guidelines on HIV Testing Services, July 2015)*

Viral nucleic acids can be detected by Nucleic Acid Amplification Tests (NAAT) like Polymerase Chain Reaction (PCR) which target the structural genes of HIV. It is particularly useful for HIV diagnosis during the window period when antibodies are absent, to resolve indeterminate serology results and for early infant diagnosis when maternal antibodies are present.

Diagnosis of HIV by detection of antibodies in serum or plasma is done using ELISA, rapid tests or Western blot (WB) following a definite algorithm. There are other approaches like Chemiluminescence Immunoassays (CLIA) and Line Immunoassays (LIA) that can also detect specific antibodies. p24 antigen can be detected using combination Enzyme Immunoassay (EIA) based systems that detect antibodies as well and is useful for diagnosis during the window period in a newly infected individual (47–49).

3.12 HIV Testing Strategies

NACO Testing Strategies

Following the detection of AIDS cases for the first time in India, in 1986 and its subsequent spread as an epidemic led to the development of the first National AIDS Control Programme (NACP) in 1992 and the formation of National AIDS Control Organization (NACO) to execute the programme. NACO functions under the Ministry of Health and Family Welfare (MoHFW) to constitute policies and implement programmes for the control and prevention of HIV infections in India (50).

The NACP-IV (2012-2017) is the programme running presently in its last year of execution. It aims to cut down new infections by 50% and provide wholesome care, support and treatment for all individuals living with HIV/ AIDS (8).

The varying prevalence of HIV in different population groups, specifically the low positive predictive value (PPV) in low prevalence populations demanded the WHO/ NACO to develop precise strategies and diagnostic algorithms depending on the diagnostic tools available in the market for the detection of HIV infection. The prevalence of HIV in a population influences the probability of a test accurately detecting the status of an individual being tested from that population group. The PPV or the probability that an individual who tested positive is truly infected, increases if the prevalence is higher.

Indian testing strategies (1, 2 and 3) include a rational sequence of performing tests serially and repeat testing originally positive samples. The tests used in the three strategies are either an ELISA or a Rapid test (E/R) and for confirmation of indeterminate or discordant results, high specificity tests like WB and Line Immunoassays (LIA) can be used.

As per the recommendations by NACO, ELISA kits with $\geq$ 99.5% sensitivity and $\geq$ 98% specificity and Rapid kits with $\geq$ 99.5% sensitivity and $\geq$ 98% specificity must be used.

The different assays used in a strategy must be based on different principles or different antigens. The first assay should have a high sensitivity and the successive assays should have a high specificity.

In case of indeterminate results, a second sample is collected 2 to 4 weeks later and should be tested by WB or PCR or referred to National Research Laboratory (NRL) for confirmation. Molecular assays may be used if the sample is repetitively giving indeterminate results (49).

3.12.1 Strategy 1

This strategy is used in transfusion and transplant screening for donor safety by performing a single test (**Figure 5**) of high sensitivity. If the sample tested is reactive then that unit is discarded, the donor is notified and referred to an Integrated Counselling and Testing Centre (ICTC) for confirmation.

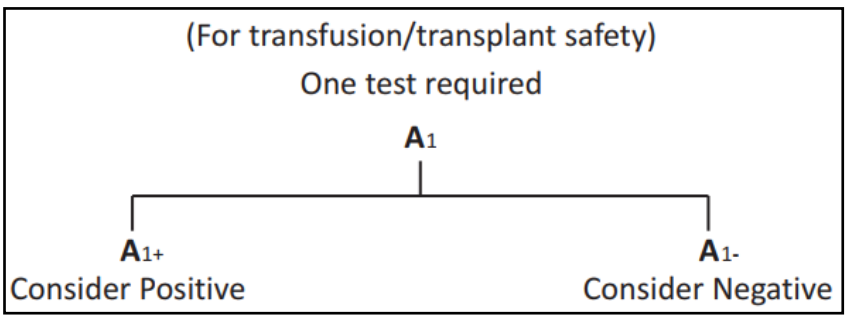


Figure 5: Strategy 1 – For blood transfusion and transplant screening

3.12.2 Strategy 2 A

This strategy is used for sentinel surveillance. If the sample that is positive by the first assay, it is repeat tested using a second different assay (**Figure 6**).

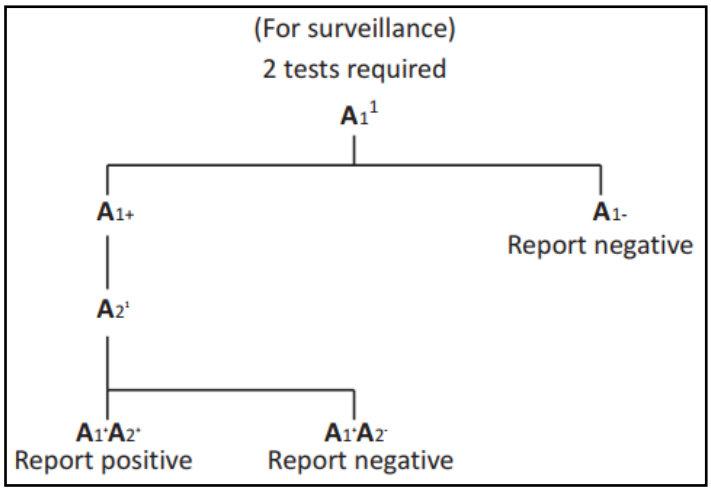


Figure 6: Strategy 2 A – For surveillance

3.12.3 Strategy 2 B

This strategy is used in individuals who are clinically symptomatic of AIDS indicator diseases. The first screening assay used is of high sensitivity. If the first two assays are positive, then it is reported as reactive. If the two assays give discordant results, then a third tie-breaker test is performed (**Figure 7**). Counselling, informed consent and confidentiality assurance is mandatory in all cases.

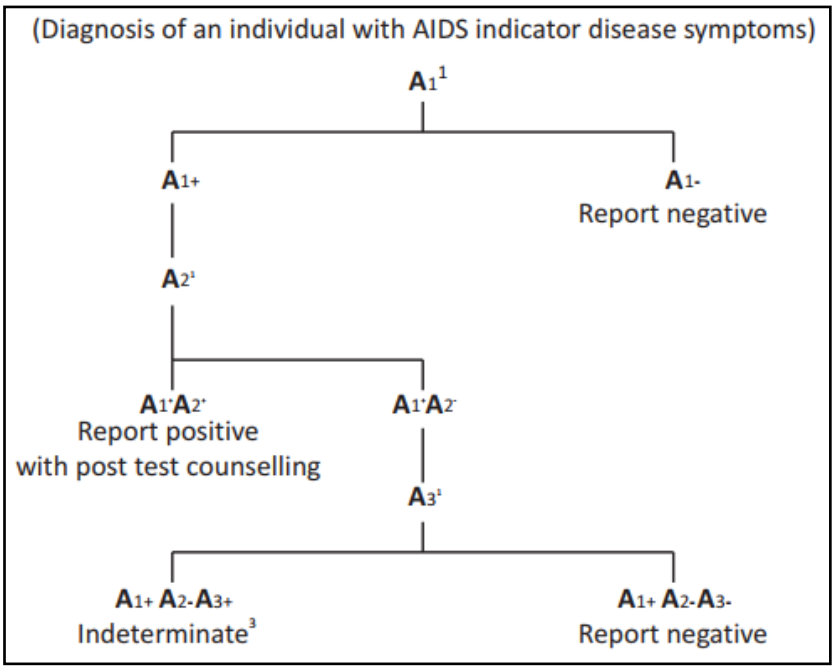


Figure 7: Strategy 2 B – For symptomatic patients with AIDS indicator disease

3.12.4 Strategy 3

This strategy is used for the diagnosis of HIV in asymptomatic individuals using an additional third test for samples which are positive by the initial test(s) as shown in **Figure 8**.

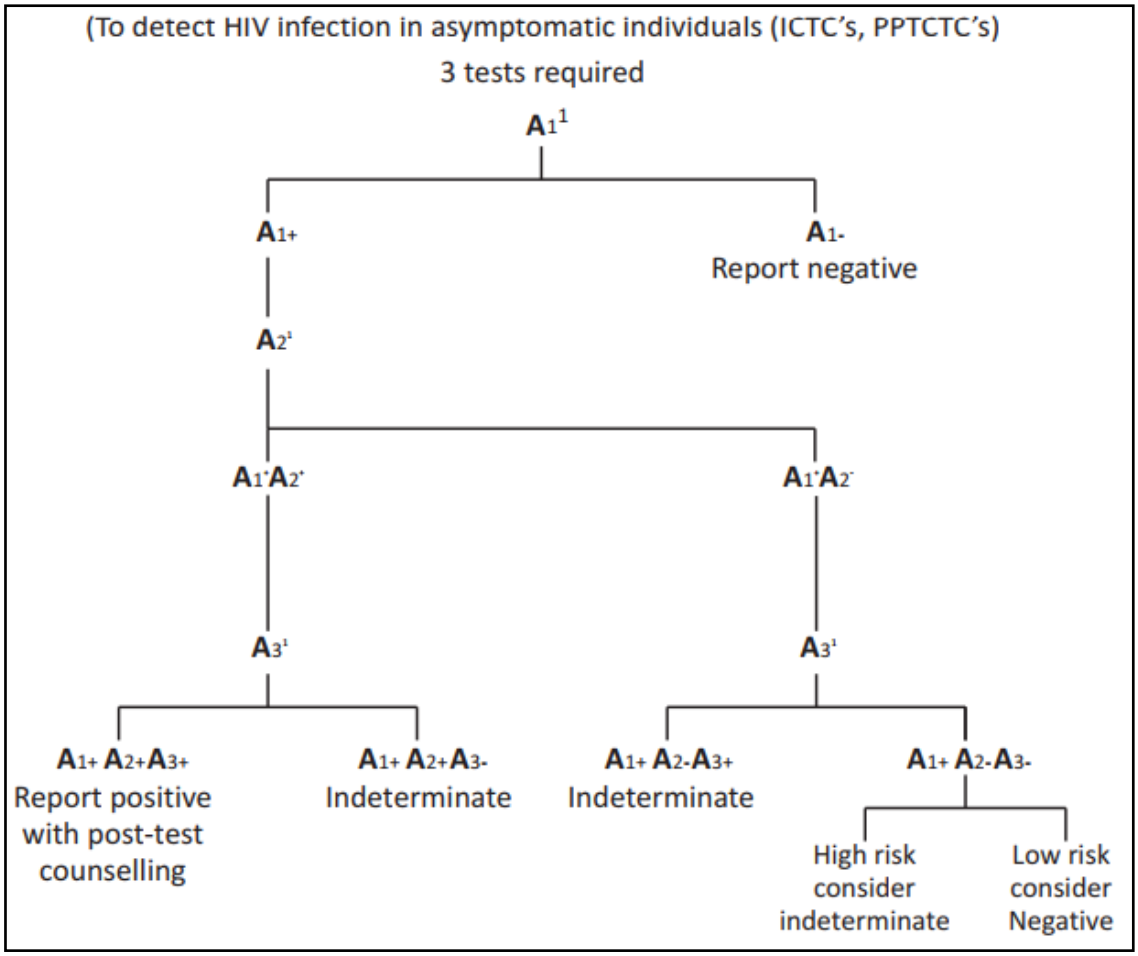


Figure 8: Strategy 3 – For testing asymptomatic individuals

CDC Testing Strategy

In 2014, CDC released HIV Diagnostic Testing Algorithm for serum or plasma samples, which was superior to the conventional strategy of HIV antibody screening followed by confirmation of positive results by Western Blot. The new algorithm helped to detect HIV infection earlier and more accurately and distinguished between HIV-1 and HIV-2 infections as well (51,52). **Figure 9** depicts the testing strategy for detection of HIV infection.

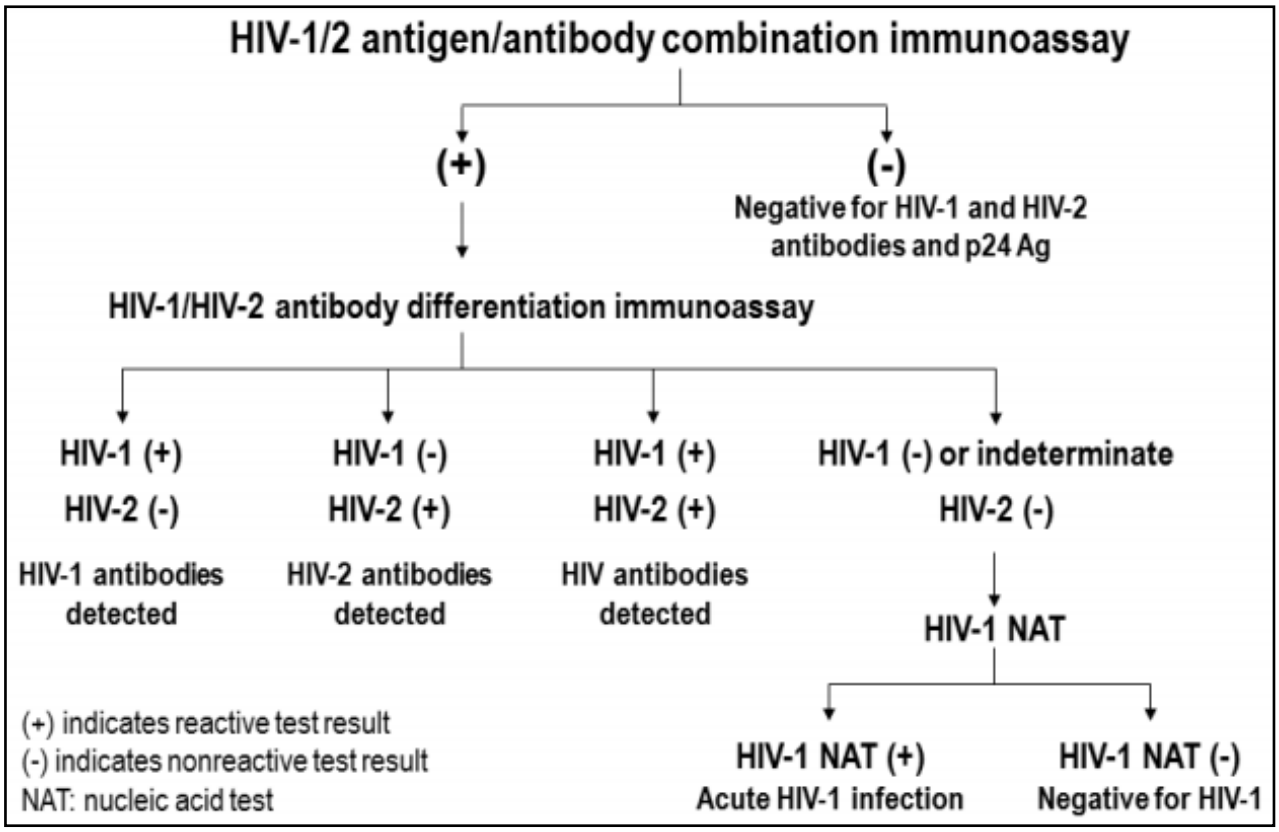


Figure 9: Recommended laboratory HIV testing algorithm

3.13 Antiretroviral therapy

3.13.1 Antiretroviral agents

Since Zidovudine, the first antiretroviral drug to be licensed became available in 1987, several newer classes of antiretroviral (ARV) drugs have been introduced with varying mechanisms of action at different steps of HIV replication (53). Science is yet to deliver a cure for HIV infection, however the use of combination antiretroviral regimens from 1996 has altered the course of the disease from a progressive fatal illness into a chronic controllable disease (31,34).

Highly active antiretroviral therapy (HAART) is the standard of care now and includes a combination of three or more anti-HIV drugs that are able to decrease viral load levels by

reducing replication and increase CD4 cell counts. It also lessens the chance of developing resistance, thus providing long term effective treatment. However, patients must receive life-long therapy in order to maintain low to undetectable viremia levels and ultimately may still develop drug resistant viral variants (34,54).

The following are the different classes of antiretroviral drugs (**Table 4**):

- Nucleoside/ nucleotide reverse transcriptase inhibitors (NRTIs): Act as normal nucleoside/ nucleotide analogues and gets inserted into the growing viral DNA chain and terminates its synthesis.
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs): Binds to HIV-1 reverse transcriptase enzyme and changes its spatial conformation, thus non-competitively inhibit reverse transcription. HIV-2 is intrinsically resistant to this class of drugs.
- Protease inhibitors (PIs): Bind to the active site of protease, the enzyme that cleaves viral polyprotein precursors during maturation of the virion.
- Integrase inhibitors: Bind to integrase enzyme-viral DNA complex and inhibit DNA strand transfer and integration into the host cell genome.
- Fusion inhibitors: Bind and disrupt transmembrane glycoprotein 41-dependent fusion of HIV virion with host cell membrane.
- CCR5 antagonists: Bind to CCR5 receptors and changes its conformation such that HIV-1 virion is unable to recognise it (54,55).

Table 4: Examples of antiretroviral agents (56,57)

Nucleoside/nucleotide analogues	Non-nucleoside RT inhibitors	Protease inhibitors	Integrase inhibitors
Zidovudine (AZT)	Nevirapine (NVP)	Saquinavir (SQV)	Raltegravir (RAL)
Lamivudine (3TC)	Efavirenz (EFV)	Lopinavir/ritonavir	Elvitegravir (EVG)
Stavudine (d4T)	Etravirine (ETR)	(LPV/r)	Dolutegravir (DTG)
Emtricitabine (FTC)	Rilpivirine (RPV)	Indinavir (IDV)	**Fusion inhibitors**
Didanosine (DDI)		Nelfinavir (NFV)	Enfuvirtide (T-20)
Abacavir (ABC)		Darunavir (DRV)	**CCR5 antagonists**
Tenofovir (TDF)		Atazanavir (ATV)	Maraviroc (MVC)
		Tipranavir (TPV)	
		Fosamprenavir (FPV)	

3.13.2 Antiretroviral Regimen

ART has shown to decelerate the disease progression and benefit in prevention of HIV transmission. This led to the U.S. Health Department to recommend ART for every individual diagnosed with HIV infection (34).

Over the years, though the WHO guidelines of when to initiate ART in HIV infected individuals have constantly evolved, yet they were limited by the individual's CD4 cell counts and clinical staging of the disease. In September 2015, WHO declared that all individuals diagnosed with HIV infection should be initiated on ART irrespective of the CD4 cell counts or clinical stage. Its 'treat all' policy made all persons living with HIV (PLHIV), regardless of population and age were eligible for therapy (58).

NACO guidelines for ART, advised initiation of therapy based on the CD4 cell count and clinical stage until May 2017, when they revised the guidelines in accordance with the WHO

recommendation and decided to treat all PLHIV with ART irrespective of CD4 count, stage, age or population (59).

Before the HIV infected person is initiated on ART he is clinically assessed to determine the stage of infection, his medical history is taken to elicit risk behaviours and a detailed physical examination is performed. Additionally a thorough laboratory evaluation is done to search for opportunistic infections and to set baseline parameters (60). Then the individual is started on first line ART regimen which usually consists of 2NRTIs + 1NNRTI (**Table 5**).

Table 5: First line regimen *(Adapted from WHO Consolidated Guidelines on the use of Antiretroviral Drugs for Treating and Preventing HIV Infections, 2nd edition, 2016)* (57)

First-line ART	Preferred first-line regimen	Alternative first-line regimens
Adults	TDF + 3TC (or FTC) + EFV	AZT + 3TC + EFV (or NVP) TDF + 3TC (or FTC) + DTG TDF + 3TC (or FTC) + EFV400 TDF + 3TC (or FTC) + NVP
Pregnant/ breastfeeding women	TDF + 3TC (or FTC) + EFV	AZT + 3TC + EFV (or NVP) TDF + 3TC (or FTC) + NVP
Adolescents	TDF + 3TC (or FTC) + EFV	AZT + 3TC + EFV (or NVP) TDF (or ABC) + 3TC (or FTC) + DTG TDF (or ABC) + 3TC (or FTC) + EFV400 TDF (or ABC) + 3TC (or FTC) + NVP
Children 3 years to < 10 years	ABC + 3TC + EFV	ABC + 3TC + NVP AZT + 3TC + EFV (or NVP) TDF + 3TC (or FTC) + EFV (or NVP)
Children < 3 years	ABC (or AZT) + 3TC + LPV/r	ABC (or AZT) + 3TC + NVP

The recent guidelines emphasizes on discontinuation of Stavudine in first-line regimen due to its metabolic toxicities. It permits the use of reduced dose Efavirenz to improve acceptability and decrease expenses. It includes integrase inhibitors in the first line drugs. Moreover it recommends the use of fixed dose combinations (FDC) and once-daily regimens for ART (57).

3.14 Monitoring

While an individual is on ART he must be monitored regularly to look for response to treatment, development of any toxicities to the ARV drugs and for improved outcomes (61).

CD4 enumeration must be done every 6 months until the patient is stable on ART. It is performed by flow cytometry using optical or electronic sensors that analyse characteristics of each cell. In order to avoid diurnal fluctuations, blood sample should be collected at similar times of the day. The specimen should not be refrigerated, instead kept at room temperature until testing, preferably immediately (49,57). Even though therapy is now initiated regardless of the CD4 count, it is still relevant in order to decide when to start or stop OI prophylaxis, to assess risk of disease progression, in priority settings to decide on ART initiation when universal treatment is not possible and finally in persons in whom ART is failing (62).

HIV viral load (VL) is the preferred method to look for patient response to therapy in routine monitoring. It is tested at 6 months, 12 months after initiating ART and every 12 months thereafter if the individual is stable on treatment. In scenarios where viral load can be routinely monitored, CD4 monitoring can be stopped in individuals with viral suppression and are stable on ART (57). In order to quantify the actual amount of HIV-1 RNA in a person, a total of cell-free virus, virus in infected cells and integrated provirus must be taken but the usual method is to measure the cell-free virus present in plasma (49). The preferred specimen for

viral load estimation is plasma, alternatively, DBS specimens prepared from capillary or venous whole blood can be used with a threshold of 1000 copies/ ml in resource-limited settings (61). Real time Reverse transcriptase-PCR (RT-PCR) is the commonest technique used and the result is reported as number of copies per ml blood. An individual who is virally suppressed must have viral load as 'undetectable' or below the lower limit of detection of the assay (49).

3.15 Treatment failure

- Virologial failure: After 6 months of initiating ART, if the VL >1000 copies/ ml in two consecutive quantifications with a 3 month interval between them during which adherence support was given.
- Clinical failure: After 6 months of receiving ART, if the individual develops new or recurrent clinical condition which indicate progressing immunodeficiency.
- Immunological failure: CD4 counts persistently <100 cells/ μl or counts ≤250 cells/ μl after clinical failure in adults and adolescents. In children <5 years, CD4 counts persistently <200 cells/ μl and in children >5 years, CD4 counts persistently <100 cells/μl.

Viral load estimation is the favoured method to identify and confirm treatment failure. A threshold of 1000 copies/ ml is recommended by WHO because below this level, the risk of transmission of infection and worsening of disease is dramatically low. Similar to routine monitoring, the samples that are used are plasma or DBS in order to extent the coverage of viral load testing to settings with infrastructural challenges.

CD4 counts and clinical assessment is used to detect treatment failure, in scenarios where viral load quantification is not done routinely. If possible, a targeted viral load testing should

be done depending on clinical and immunological benchmarks to confirm virological failure and thus avoid unnecessary switching of treatment regimen to second-line ART (57,61).

The algorithm that is followed to decide whether a change in treatment regimen is warranted, is shown in **Figure 10**.

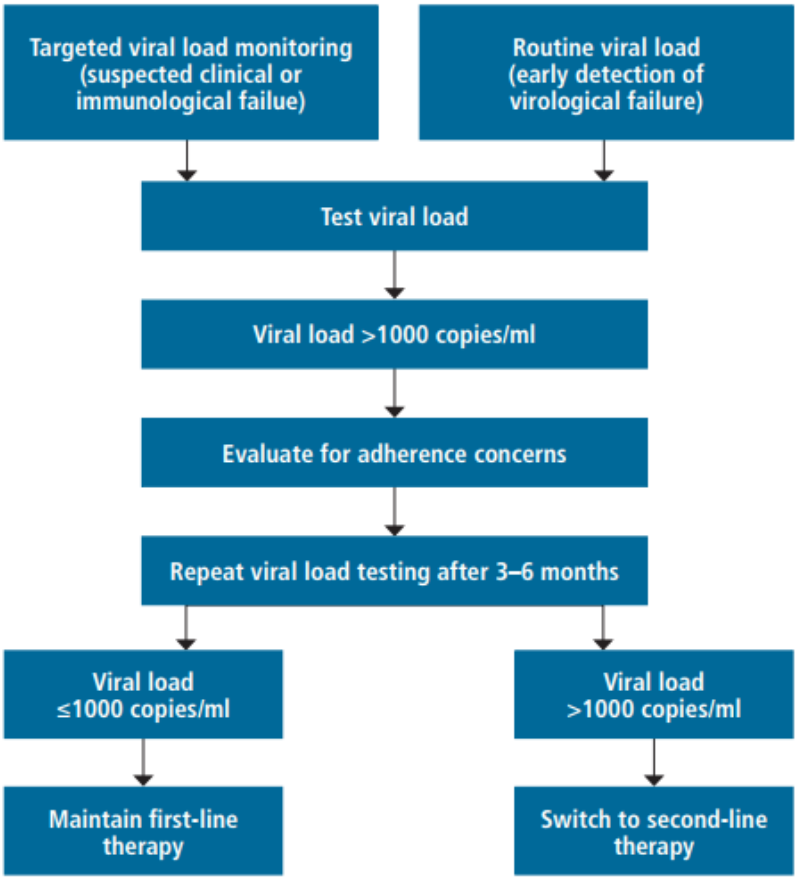


Figure 10: HIV-1 viral load estimation strategy *(Adapted from WHO Information Note on HIV Treatment and Care, July 2017 Update)*

3.16 HIV-1 Drug resistance

Under the aegis of WHO and NACO there has been a steady upsurge in PLHIV receiving ART, worldwide and in India, respectively. ART has proven to diminish HIV-associated morbidity and mortality, however, poor adherence and suboptimal treatment can result in incomplete viral suppression which inevitably leads to advent of drug-resistant strains.

Individuals receiving ART are under the constant threat of resistance and subsequent treatment failure, and also the transmission of resistant strains is of rising concern (54,63,64).

3.16.1 Evolution of drug resistance patterns

In the initial decade of ART, single and dual drug regimens mainly consisting of Zidovudine (AZT) and Stavudine (d4T) were used. However, the development of resistance to these first regimens led to the pursuit of newer effective regimens. The resistant strains selected out by the extensive use of NRTI, gave rise to cross-resistance to other members of the same class, to which there was no previous exposure (54,65). The addition of PI and NNRTI to those resistant to NRTI was beneficial clinically but resistance to these newer drugs led to triple-class resistance (65).

There has been a paradigm shift in resistance patterns since a NNRTI was added to 2 NRTI in the first-line regimen and AZT and d4T were replaced by Tenofovir (TDF) and Abacavir (ABC). These regimens have brought about better virological suppression and improved tolerability. As a result, the commonest resistance in treatment failure is seen against NNRTI (Efavirenz or Nevirapine) and Lamivudine or Emtricitabine (66). Likewise, ritonavir-boosted PI has shown to have an upper hand over non-boosted PI with enhanced virological potency. Unlike other classes of drugs, resistance to PI is rarely detected among individuals on boosted PI-based regimens in treatment failure probably due to their high genetic barrier (65,67).

3.16.2 Epidemiology

Currently, most of the data available on HIV-1 drug resistance is on subtype B, despite the fact that non-B subtypes cause majority of the global infections (68). The WHO pre-treatment drug resistance (PDR) surveillance data from 2014-2016, point out that NNRTI resistance has increased to >10% in six of the 11 countries surveyed from Africa, Asia and Latin America.

And the overall prevalence of NNRTI resistance among ART-experienced individuals ranged from 4% to 28% in Africa, where the major subtype is C (69). According to NACO, out of the 10.75 lakh individuals on ART, 25000 are on second-line regimen and 450 on third-line regimen following treatment failure to first-line and second-line drugs, respectively. National AIDS Research Institute (NARI), Pune has recently taken on the responsibility of conducting a nation-wide survey to gauge the burden of HIV drug resistance in India (70).

3.16.3 Types of resistance

A characteristic feature of HIV infection is its high level of replication and turnover in infected individuals. Furthermore, the notoriously error-prone nature of reverse transcription, with an average of one mutation per each transcribed viral genome, results in a highly heterogeneous viral population in an infected individual. These two factors together contribute to the patient having an assorted mixture of viral quasispecies. Any of these mutations generated in the presence of ARV drugs, may grant the virus a selective advantage of reduced susceptibility to ARV agents. The respective viral quasispecies will surpass the others in accordance with the Darwinian selection process. This *induced* or *acquired resistance*, is one mode of developing drug resistance (54).

Alternatively, individuals may be primarily infected with HIV strains that are resistant to single or multiple ARV agents. As the prevalence of drug resistant HIV-1 rises among infected individuals, the risk of transmission of resistant viruses to newly infected also increases (65,71). This *transmitted drug resistance (TDR)*, affects ART outcomes adversely by a faster rate of virological failure when compared to persons infected with susceptible strains. Accordingly, developed countries recommend resistance testing prior to ART

initiation. However, in developing countries with an extended access to ART and where non-B subtypes predominate, pre-ART resistance testing is not available for routine use (72).

Though WHO recognises the importance of resistance testing for patients to guide selection of ART regimen, it presently does not recommend it routinely. For the purpose of policy making, it recommends surveillance of HIV drug resistance (57) by detection of any one of the surveillance drug resistant mutations (SDRMs) given in WHO surveillance mutation list (72,73).

3.16.4 Genetic barrier to resistance

The number of mutations required to cause resistance to a particular ARV, and the frequency at which it occurs, decides the 'genetic barrier to resistance' of that ARV. Some drugs which require multiple drug resistance mutations to cause reduced susceptibility have a high genetic barrier to resistance, whereas others which may need only a single mutation have a low genetic barrier to resistance.

Drug resistance mutations may either be primary mutations, which act directly to reduce the susceptibility of HIV to an ARV, or accessory mutations, which promote viral fitness and thus reduce susceptibility. Also the inherent antiviral potency of different ARV vary considerably. Both the genetic barrier to resistance and the inherent antiviral potency determines the vulnerability of an ARV to resistance (74). The relative genetic barriers and potencies of each class of ARVs are depicted in **Figure 11**.

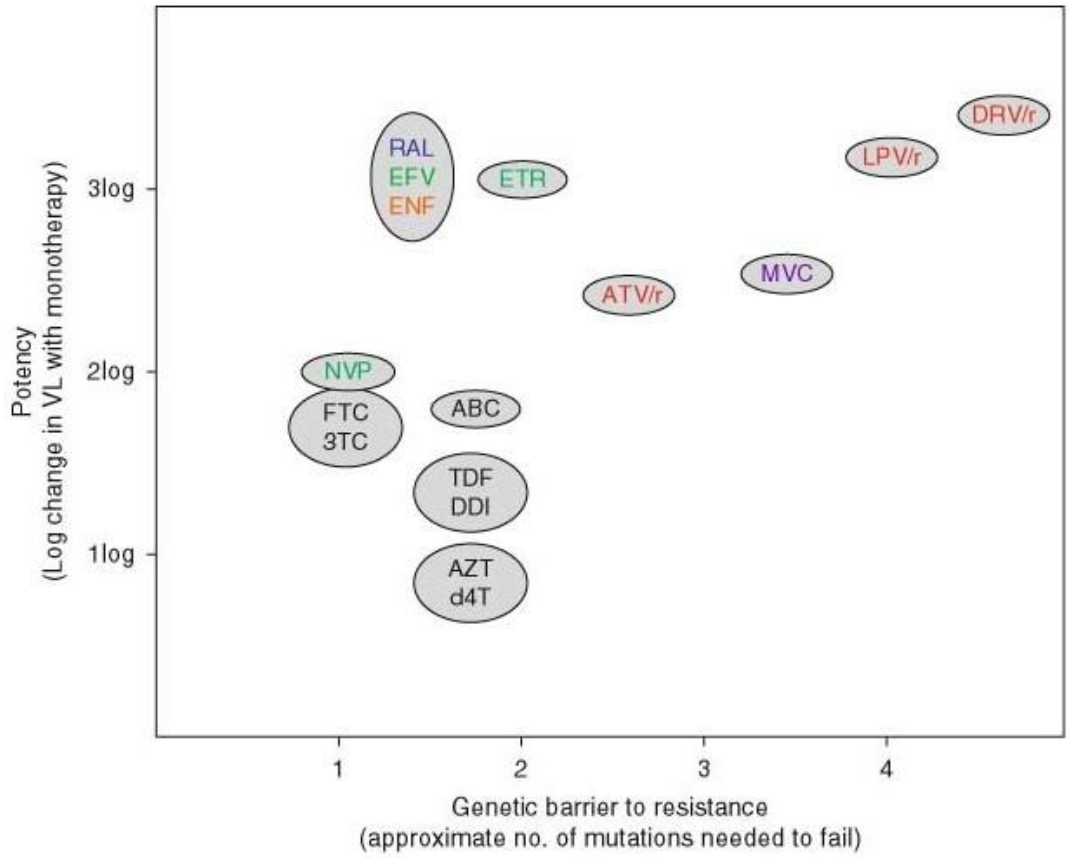


Figure 11: Diagrammatic representation of genetic barrier to resistance and potency or antiviral activity of drugs from each ARV class. d4T = Stavudine, AZT = Zidovudine, DDl = Didanosine, TDF = Tenofovir, 3TC = Lamivudine, FTC = Emtricitabine, ABC = Abacavir, NVP = Nevirapine, EFV = Efavirenz, ETR = Etravirine, ATV/r = ritonavir boosted Atazanavir, LPV/r = ritonavir boosted Lopinavir, DRV/r = ritonavir boosted Darunavir, RAL = Raltegravir, ENF = Enfuvirtide, MVC = Maraviroc. *(Adapted from Tang MW, et al. HIV-1 Antiretroviral Resistance. Drugs. 2012)*

3.16.5 HIV-1 drug resistance testing

The progress of drug resistance has substantial implications while choosing ARV regimens. The accumulation of mutations within the reverse transcriptase (RT) gene, which is the target for 2 major classes of ARV drugs, has led to a point where drug resistance testing must become an essential part of HIV care, possibly at the time of HIV diagnosis and compulsory in all cases of virological failure (75).

Resistance testing can be done either by phenotypic or genotypic methods, to assess infecting virus strains. Genotyping detects resistance causing mutations and phenotypic assays are essentially drug susceptibility tests in which a fixed inoculum of the virus is grown in the presence of serial dilutions of the drug. Both the tests include extraction of the virus from plasma, reverse transcription of contiguous protease (PR) and RT genes, and amplification by PCR (76). These assays detect resistance to NRTIs, NNRTIs and PIs. Testing for integrase inhibitor resistance and fusion inhibitor resistance may have to be ordered separately. And co-receptor tropism assays should be performed prior to use of CCR5 antagonist (77).

3.16.5.1 Phenotypic testing

Phenotypic *in vitro* susceptibility assays test the ability of the virus to grow in cell culture at different drug concentrations. It is typically reported as the drug concentration that inhibits 50% (IC_{50}) of HIV virus replication. A ratio obtained by comparing the IC_{50} of the test virus to that of a drug susceptible reference HIV strain is referred to as fold increase or fold change in IC_{50}. These assays use recombinant viruses generated by introducing PCR-amplified segments (PR/RT, integrase or envelope gene sequences) of patient virus genome extracted from plasma into a HIV wild type laboratory construct (78,79).

The advantages of phenotypic testing is that it directly measures drug susceptibility which is cumulative of the acquired mutations in the test strain. This technique is necessary to establish genotype-phenotype correlations for the development of new ARV drugs and salvage regimens in highly treated patients infected with virus strains having multiple mutations (76,80).

As a result of the high cost, longer turnaround time, insensitivity to minor viral variants and lack of cut-offs for clinical resistance among all ARV agents, phenotypic testing is mainly set aside for drug resistance research (76,79).

3.16.5.2 Genotypic testing

It detects drug resistance mutations present in pertinent viral genes. Following the extraction of the virus from plasma, reverse transcription of the complete PR gene and most of RT gene and amplification by PCR, it is finally conventionally sequenced and its nucleotide sequence is analysed to detect mutations known to confer resistance. Since there are over 150 recognised mutations associated with HIV drug resistance (HIVDR) and several interactions between the mutations, the analysis of a resistance profile is complex. Hence various genotypic resistance interpretation algorithms have been developed. The International AIDS Society-USA (IAS-USA) has a list of mutations in the RT, PR, integrase and envelope genes. The WHO HIVResNet also has a list of noteworthy mutations suitable for surveillance of transmitted DR-HIV. The Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu) also guides the evaluation of genotypic test results.

Some of the issues with resistance testing are that it is not performed everywhere, only in selected reference laboratories and are quite expensive. The tests do not detect minority mutations and usually do not work if the sample has a VL < 1000 copies/ ml. And comprehending the results of the test can be challenging (81).

Nonetheless, genotypic assays can be completed quickly and the report may be offered within 1-2 weeks of collecting the sample (77,82). The WHO has conventionally recommended plasma which has been separated from an anticoagulated tube of blood, as the sample for genotyping. Separation and storing of the plasma must be done within 6 hours of sample

collection and frozen plasma specimens need to be shipped on dry ice. Successful amplification from plasma depends on viral load, time interval between blood collection and plasma separation, nature of plasma (haemolysis), and time interval from separation to storage, storage temperature and time taken prior to testing. Therefore, only settings which are able to correctly process and ship plasma samples must use it for HIV genotyping.

Serum can be collected as a specimen, following all the precautions taken for plasma. However, studies have shown that viral load in serum is markedly lower than in plasma.

Dried blood spots (DBS) can also be used for HIV drug resistance genotyping, by preparing it with blood drawn for routine purposes or surveillance and it does not require any special processing (82).

The concept of spotting blood on a filter paper and then utilising it for diagnostic purposes began almost a century ago. The key attributes of DBS which makes it advantageous over routine samples were described by Chapman in 1924. They hold good even today and are:

- A lesser volume of blood is required when compared to conventional phlebotomy.
- Collection of blood is easy, non-invasive and economical.
- There is minimal chances of bacterial contamination or haemolysis.
- Much longer durations of storage is possible with DBS, with almost no degradation of the analytes.

For many years, DBS was primarily used in resource-limited situations for the serological diagnosis of infectious diseases like syphilis, mumps, measles, poliovirus, respiratory syncytial virus and parainfluenza virus, for the direct detection of Shigella from faeces samples dried onto filter paper and in the efficient screening for inherited metabolic diseases

in neonates. Later, from 2005 onwards a whole range of novel and innovative applications for DBS have opened up (83). For the detection of infectious diseases by serology or molecular methods, DBS can be relied upon, since antibodies and nucleic acids remain stable for longer periods when compared to whole blood, plasma or serum (84).

In the market, very many types of filter paper brands are available with varying thickness and pore sizes. The two main brands that are approved by US Food and Drug Administration (FDA) for human whole blood collection are Whatman 903 and PerkinElmer 226 filter paper cards, which show minimal difference in detection of analytes. There are also treated filter paper cards available, FTA Elute and FTA (Whatman; GE Healthcare, UK), which inactivate antibodies, viruses and bacteria and causes cell lysis. Such cards can be used only for Nucleic Acid Amplification Tests (NAATs) (85).

DBS appears to be an attractive alternative to plasma samples for HIV-1 drug resistance testing. It offers a useful and dependable way to obtain, store and transport blood samples to reference laboratories offering drug resistance testing, which is essential to developing cost-effective assays in resource-limited settings. Whole blood from a finger or heel stick puncture can be effortlessly collected onto a filter paper, thus presenting a technical and monetary benefit over conventional phlebotomy. Since the sample collection is easier, it avoids the use of syringes and vacutainer tubes, decreases the biohazard risk to the phlebotomist and the need for centrifugation. HIV-1 loses its infectivity due to the disruption of its envelope on drying. DBS samples are thus non-infectious and can be readily dispatched in sealed envelopes to higher centres, whereas, plasma needs to be transported in break-proof containers and requires dry ice to preserve the cold-chain. This in turn will add substantial bulk to the item and necessitate specialized handling (15,86).

The WHO working along with specialists has provided a reference protocol on DBS preparation, storage and transport conditions and processing for HIV-1 drug resistant genotypic testing (87).

Drug resistance studies from India, where subtype C is prevalent, is the need of the hour. Specifically, the stability of DBS at the tropical temperature in our country should be looked into. Then DBS can be used as the convenient and economical sample in India, thus helping the resource-limited settings avail the ideal tools needed in the management of HIV infection.

3.16.6 Minority and archived viral populations

The prevailing population of resistant virus in plasma does not represent the heterogeneous viral quasispecies in individuals failing HAART. Smaller populations of virus with distinct mutations can serve as a reservoir for novel resistant genotypes and throughout the HIV infection, viral genomes are endlessly being archived as latently integrated proviruses (54). Conventional genotyping techniques, merely detects variants with a frequency ≥ 20% in an infected individual. Nevertheless, novel assays like next generation sequencing (NGS) can detect minority variants at frequencies that are considerably low. These technologies help to identify the actual rate of drug resistant variants in treatment-naïve and -experienced persons and gains significance, since pre-existing resistant minority variants can jeopardize subsequent treatment (68).

As the half-life of HIV in plasma is around 6 hours, only actively replicating virus can be obtained and the resulting sequence represents the latest quasispecies selected by ART. However, the proviral DNA in PBMC may contain multiple archived mutations that are absent in plasma (76) making PBMC, a probable complimentary sample to plasma.

4. Materials and Methods

This study was done in the Departments of Clinical Virology and Clinical Microbiology, Christian Medical College and Hospital, Vellore. It was approved by the Institutional Review Board (Reference IRB Min. No. 9832 dated 07.01.2016) and was funded by Fluid Research Fund (Account no. 22Y966) and Virology Special Fund.

4.1 Materials

4.1.1 Study subjects

Consecutive HIV-1 infected individuals in treatment failure referred for HIV-1 drug resistance genotyping were recruited for the study and their plasma, DBS and PBMC samples were tested for HIV-1 drug resistance mutations. The study was explained to all the participating individuals and were recruited only after getting an informed consent. This study was conducted over a period of 14 months (July 2016 – August 2017).

Inclusion criteria:

1) Serologically confirmed HIV-1 infected individuals
2) ART-experienced individuals with clinical, immunological and/or virological failure (>1000 copies/ml)
3) Individuals above 18 years of age
4) Individuals who consent to the study

Exclusion criteria:

1) HIV-1 negative plasma samples by reference standard
2) ART-naïve individuals

3) Individuals under 18 years of age

4) Individuals who do not consent to the study

4.1.2 Sample size

The prevalence of HIV-1 infection in India is 0.3 with 5% individuals developing drug resistance following initiation of treatment. Thus the HIV-1 drug resistance prevalence was calculated to be around 0.02. The required sample size to show an agreement of 0.9 with a prevalence of HIV-1 drug resistance of 2%, with 80% power and 5% level of significance was found to be 34 HIV infected individuals. However, it was possible to recruit only 29 individuals.

Agreement -Single group- Dichotomous outcome-Kappa (88)

(Testing against Population value)

Population agreement	0.05
Sample agreement	0.9
Prevalence (Proportion)	0.02
Power (1-beta)	80
Alpha error (%)	5
1 or 2 sided	2
Required sample size	34

Formula:

$$n = \frac{\left(z_{\alpha} + z_{1-\beta}\right)^2}{\left\{\pi(1-\pi)(\rho_1 - \rho_0)\right\}^2 \left[\frac{1}{\pi^2 + \pi(1-\pi)\rho_0} + \frac{2}{\pi(1-\pi)(1-\rho_0)} + \frac{1}{(1-\pi)^2 + \pi(1-\pi)\rho_0}\right]}$$

Where,

ρ_0 : Null hypothesis Agreement

ρ_1 : Alternative hypothesis agreement

π : Prevalence

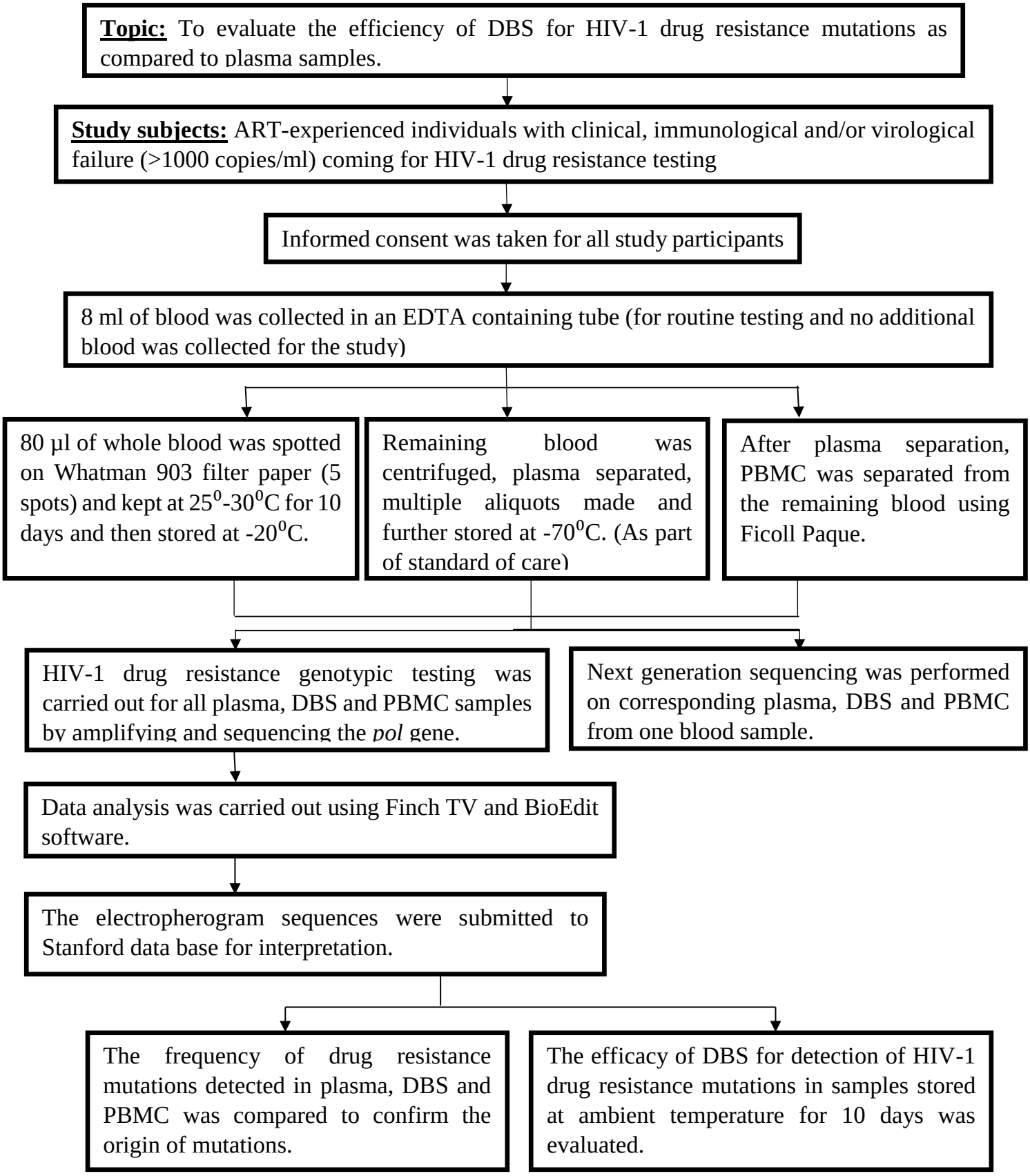
α : Significance level

1- β : Power

4.1.3 Specimen collection

After obtaining an informed consent, 8 ml of blood was collected in a sterile EDTA containing tube for routine testing; no additional blood was collected for the study. 5 spots, each of 80µl of whole blood was spotted on Whatman 903 filter paper card and kept at 25^0-30^0C for 10 days and then stored at -20^0C until the time of testing. The remaining blood was centrifuged, plasma separated, multiple aliquots made and then stored at -70^0C until testing, as part of standard of care. After plasma was separated and aliquoted, PBMC was extracted from the remaining blood and stored at -70^0C until it was tested.

4.1.4 Study algorithm



4.2 Methods

4.2.1 Sample preparation:

Plasma (63,89)

Plasma was separated from the EDTA tubes after they were centrifuged at 2000 rpm for 10 minutes at 4^0C. The plasma was stored as multiple aliquots at -70^0C until the time of testing.

DBS

80µl each of whole blood was spotted on to Whatman 903 filter paper card as 5 spots, dried overnight, covered in butter paper and put into a plastic zip lock pouch with two desiccants and kept at 25^0-30^0C for 10 days and then stored at -20^0C until testing was done by taking punches of a spot.

PBMC

PBMC was separated from the remaining blood after spotting the filter paper card and plasma separation, by the following method. Three ml of blood sample was diluted in 6 ml of 1X phosphate buffered saline (PBS) in a sterile 15 ml tube. The 9 ml of diluted blood sample was mixed well in a plate mixer and 3 ml each of diluted blood samples was carefully overlain into another 3 sterile 15 ml tube containing 3ml of Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Then the tubes were centrifuged at 2000 rpm for 20 minutes at 20^0C and after centrifugation the PBMC cell layer formed at the centre of the tubes was carefully transferred into another sterile15 ml tube. This tube was centrifuged at 2000 rpm for 10 minutes at 20^0C to pellet the PBMCs. 1 ml 1X RBC lysis buffer was added to the PBMC pellet, mixed and incubated for 1 minute at room temperature. The PBMCs were washed with 4ml 1X PBS and centrifuged again for 10 min at 2000 rpm. This washing step was repeated using 5 ml of 1X PBS. After centrifugation, 1.5 ml 1X PBS was added to the PBMCs cells and counted using

the Beckman coulter UniCel DxH 800 (Miami, USA) in the Department of Clinical Pathology. Then the PBMCs cells were aliquoted based on their cell counts into sterile multiple tubes and overlaid with RNA Later and stored at -70°C until testing.

4.2.2 Extraction of nucleic acids:

Plasma

HIV-1 RNA was extracted from the plasma using QIAamp® viral RNA extraction reagents (Qiagen GmbH, Hilden, Germany). The RNA extraction protocol was as follows:

1) Pipet 560 µl of lysis buffer AVL into a 1.5 ml microcentrifuge tube.
2) Add 140 µl of plasma sample to the buffer AVL in the microcentrifuge tube. Pulse-vortex for 15 seconds and centrifuge at 8000 rpm for 1 minute.
3) Incubate at room temperature (15°C-25°C) for 10 minutes to ensure complete lysis.
4) Add 560 µl of ethanol (96-100%) to the sample. Pulse-vortex for 15 seconds and centrifuge at 9000 rpm for 30 seconds.
5) Transfer 630 µl of the solution from Step 4 to the QIAamp spin column without wetting the rim. Close the spin column and centrifuge at 8000 rpm for 1 minute.
6) Place the QIAamp spin column into a fresh 2 ml collection tube and discard the tube containing the filtrate.
7) Add the remaining 630 µl to the QIAamp spin column. Spin at 8000 rpm for 1 minute. Place the QIAamp spin column into a fresh 2 ml collection tube and discard the tube containing the filtrate.
8) Add 500 µl of wash buffer AW1 and centrifuge at 8000 rpm for 1 minute. Transfer the QIAamp spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.

9) Add 500 µl of wash buffer AW2 and centrifuge at 14000 rpm for 3 minutes.
10) Transfer the QIAamp spin column into a clean 2 ml collection tube and discard the tube containing the filtrate. Centrifuge at 14000 rpm for 1 minute.
11) Place the QIAamp spin column into a clean 1.5 ml microcentrifuge tube and discard the previous collection tube containing the filtrate. Add 50 µl elution buffer. Incubate at room temperature for 1 minute and then centrifuge at 8000 rpm for 2 minutes.

DBS

HIV-1 DNA was extracted from DBS using QIAamp® DNA Mini extraction reagents (Qiagen GmbH, Hilden, Germany). The extraction protocol was as follows:

1) Take 3 punched out circles from a dried blood spot with a single-hole paper puncher into a 1.5 ml microcentrifuge tube and add 180 µl of lysis buffer ATL.
2) Incubate at 85^0C for 10 minutes. Spin down to remove drops from inside the lid.
3) Add 20 µl of proteinase K and mix by vortexing. Incubate at 56^0C for 1 hour and centrifuge to remove drops from inside the lid.
4) Add 200 µl lysis buffer AL to the sample and mix thoroughly by vortexing. Incubate at 70^0C for 10 minutes and centrifuge to remove drops from inside the lid.
5) Add 200 µl of ethanol (96-100%) and mix well by vortexing. Briefly centrifuge to remove drops from inside the lid.
6) Transfer the solution from Step 5 in to the QIAamp Mini spin column without wetting the rim. Close the cap of the spin column and centrifuge at 8000 rpm for 1 minute. Place the QIAamp Mini spin column in a fresh 2 ml collection tube and discard the tube containing the filtrate.

7) Add 500 µl of wash buffer AW1 and centrifuge at 8000 rpm for 1 minute. Transfer the QIAamp Mini spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.

8) Add 500 µl of wash buffer AW2 and centrifuge at 14000 rpm for 3 minutes.

9) Transfer the QIAamp Mini spin column into a clean 2 ml collection tube and discard the tube containing the filtrate. Centrifuge at 14000 rpm for 1 minute.

10) Place the QIAamp Mini spin column into a clean 1.5 ml microcentrifuge tube and discard the previous collection tube containing the filtrate. Add 50 µl elution buffer AE. Incubate at room temperature for 1 minute and then centrifuge at 8000 rpm for 1 minute.

PBMC

HIV-1 DNA was extracted from PBMC using QIAamp® DNA Blood Mini extraction kit (Qiagen GmbH, Hilden, Germany). The extraction protocol was as follows:

1) Discard the RNA Later and re-suspend the PBMC in 200 µl 1X PBS. Pulse-vortex to ensure thorough mixing of the cells.

2) Take 20 µl QIAGEN Protease in a 1.5 ml microcentrifuge tube. Add 200 µl sample (5 x 10^6 lymphocytes in 200 µl PBS) to the microcentrifuge tube.

3) Add 200 µl lysis buffer AL to the sample and mix well by pulse vortexing.

4) Incubate at 56^0C for 10 minutes. Briefly spin down to remove the drops from inside the lid of the microcentrifuge tube.

5) Add 200 µl ethanol (96-100%) to the sample and mix by pulse-vortexing. Briefly centrifuge to remove the drops from inside the lid of the microcentrifuge tube.

6) Transfer the solution from Step 5 in to the QIAamp Mini spin column without wetting the rim. Close the cap of the spin column and centrifuge at 8000 rpm for 1 minute. Place

the QIAamp Mini spin column in a fresh 2 ml collection tube and discard the tube containing the filtrate.

7) Add 500 µl of wash buffer AW1 and centrifuge at 8000 rpm for 1 minute. Transfer the QIAamp Mini spin column into a clean 2 ml collection tube and discard the tube containing the filtrate.

8) Add 500 µl of wash buffer AW2 and centrifuge at 14000 rpm for 3 minutes.

9) Transfer the QIAamp Mini spin column into a clean 2 ml collection tube and discard the tube containing the filtrate. Centrifuge at 14000 rpm for 1 minute.

10) Place the QIAamp Mini spin column into a clean 1.5 ml microcentrifuge tube and discard the previous collection tube containing the filtrate. Add 100 µl elution buffer AE. Incubate at room temperature for 1 minute and then centrifuge at 8000 rpm for 1 minute.

4.2.3 HIV-1 *pol* gene amplification

Plasma

The genotypic resistance assay was carried out by sequencing the Protease (PR) and Reverse transcriptase (RT) genes. The extracted RNA (20µl) was subjected to a one step RT-PCR (Qiagen GmbH, Hilden, Germany) using HotStart Taq DNA polymerase for the amplification of cDNA with specific forward and reverse primers, HIV-1 out 1 and HIV-1 out 2 respectively (custom synthesized at Invitrogen, CA, USA). The primer sequences are shown in **Table 6**.

Table 6: Outer primer sequences

Primer	Sequence
Outer forward	AATGATGACAGCATGTCAGGGAGT
Outer reverse	AGTCTTTCCCCATATTACTATGCTTTC

The reaction mix and the cycling conditions for the 1st round of amplification are shown in **Tables 7** and **8**, respectively.

Table 7: Reaction mix for the first round of amplification

Ingredients	**For 1 reaction (µl)**
5X Buffer	10
dNTPs	2
OUT-1	1
OUT-2	1
Enzyme	2
Milli Q water	14
Extracted RNA	20
Total volume	**50 µl**

Table 8: Cycling conditions for the first round of amplification

Cycle temperature	**Cycle time**	**Number of cycles**
50°C	30 minutes	1
95°C	15 minutes	1
94°C	45 seconds	40
60°C	45 seconds	40
72°C	2 minutes	40
72°C	7 minutes	1

The first round products were further amplified using Qiagen Hot Start Master Mix (Qiagen GmbH, Hilden, Germany) with forward and reverse primers, PCR res 1 and PCR res 2 respectively. The primer sequences are shown in **Table 9**.

Table 9: Inner primer sequences

Primer	Sequence
Inner forward	GGAAAAAGGGCTGTTGGAAATGTG
Inner reverse	GGCTCTTGATAAATTTGATATGTCCATTG

The reaction mix and the cycling conditions for the 2nd round of amplification are shown in **Tables 10** and **11**, respectively.

Table 10: Reaction mix for the second round of amplification

Ingredients	For 1 reaction (µl)
Hot Start Master Mix	25
Res-1	0.5
Res-2	0.5
Milli Q water	22
1st round product	2
Total volume	**50 µl**

Table 11: Cycling conditions for the second round of amplification

Cycle temperature	Cycle time	Number of cycles
95°C	15 minutes	1
94°C	45 seconds	23
55°C	45 seconds	23
72°C	2 minutes	23
72°C	7 minutes	1

The amplification reactions were carried out on Veriti (Applied Biosystems, California, USA) or GeneAmp (Applied Biosystems, Foster City, California, USA).

The amplified products were run on an ethidium bromide-stained 2% agarose (Sigma Aldrich Inc., St Louis, MO, USA) gel to check for the specific 1800 base pair size amplicon. The agarose gel was visualized using the gel documentation system Geldoc 2000 (BioRad, CA, USA) using the software Quantity One version 4.1.1 (BioRad, CA, USA).

DBS and PBMC

Since QIAamp® DNA Mini extraction kit co-purifies DNA and RNA, both the nucleic acids were amplified from DBS – RNA, by the same protocol as for *pol* gene amplification from plasma and DNA amplification was done as follows:

The genotypic resistance assay was carried out by sequencing the Protease (PR) and Reverse transcriptase (RT) genes. The extracted DNA was amplified using Qiagen Hot Start Master Mix (Qiagen GmbH, Hilden, Germany) with specific forward and reverse primers, HIV-1 out 1 and HIV-1 out 2 respectively (custom synthesized at Invitrogen, CA, USA).

The reaction mix and the cycling conditions for the 1st round of amplification are shown in **Tables 12** and **13**, respectively.

Table 12: Reaction mix for the first round of amplification

Ingredients	**For 1 reaction (µl)**
Hot Start Master Mix	25
OUT-1	0.25
OUT-2	0.25
Milli Q water	14.5
Extracted DNA	10
Total volume	**50 µl**

Table 13: Cycling conditions for the first round of amplification

Cycle temperature	Cycle time	Number of cycles
95°C	15 minutes	1
94°C	45 seconds	45
55°C	45 seconds	45
72°C	2 minutes	45
72°C	7 minutes	1
4°C	∞	

The first round products were further amplified using Qiagen Hot Start Master Mix (Qiagen GmbH, Hilden, Germany) with forward and reverse primers, PCR res 1 and PCR res 2 respectively. The reaction mix and the cycling conditions for the 2nd round of amplification are shown in **Tables 14** and **15**, respectively.

Table 14: Reaction mix for the second round of amplification

Ingredients	For 1 reaction (µl)
Hot Start Master Mix	25
Res-1	0.25
Res-2	0.25
Milli Q water	19.5
1st round product	5
Total volume	**50 µl**

Table 15: Cycling conditions for the second round of amplification

Cycle temperature	Cycle time	Number of cycles
95°C	15 minutes	1
94°C	45 seconds	40
55°C	45 seconds	40
72°C	2 minutes	40
72°C	7 minutes	1
4°C	∞	

The amplification reactions were carried out on Veriti (Applied Biosystems, California, USA) or GeneAmp (Applied Biosystems, Foster City, California, USA).

The amplified products were run on an ethidium bromide-stained 2% agarose (Sigma Aldrich Inc., St Louis, MO, USA) gel to check for the specific 1800 base pair size amplicon. The agarose gel is visualized using the gel documentation system Geldoc 2000 (BioRad, CA, USA) using the software Quantity One version 4.1.1 (BioRad, CA, USA).

4.2.4 Modifications for unamplified samples:

4.2.4.1 Modifications in extraction:

Plasma

- Instead of 140 µl, an initial volume of 420 µl of plasma was used for extraction along with proportionally increased reagents for samples with low viral load levels.

DBS

- Instead of 3 punched out circles, 6 punches were used for extraction from unamplified samples with lower viral load and 1 punched out circle was taken for unamplified samples with high viral load.

- After the addition of 50 µl elution buffer AE, incubation time was increased to 5 minutes from 1 minute.

PBMC

- In unamplified samples, 2 aliquots of PBMC were taken and re-suspended in 200 µl 1X PBS and used for extraction.

4.2.4.2 Modifications in amplification:

Plasma

- In the second round of amplification, the volume of the first round product used was increased from 2 µl to 5 µl along with a reduction in the volume of Milli Q water from 22 µl to 19 µl.
- If the sample still did not amplify, the volume of the first round product used was further increased to 10 µl along with a reduction in the volume of Milli Q water to 14 µl.

DBS

- For unamplified samples with low viral load, in the first round of DNA amplification, the volume of DNA extract used was increased from 10 µl to 20 µl along with reduction in the volume of Milli Q water from 14.5 µl to 4.5 µl. And subsequently in the second round of amplification, the volume of first round product used was increased from 5 µl to 10 µl with reduction in the volume of Milli Q water used to 14.5 µl.
- For unamplified samples with high viral load, in the first round of DNA amplification, the volume of DNA extract used was decreased from 10 µl to 5 µl along with increase in the volume of Milli Q water from 14.5 µl to 19.5 µl.

- If the high viral load samples still did not amplify, then the DNA extract was diluted using nuclease-free water in equal parts or in 1:10 ratio and then used for the first round of amplification.

PBMC

- For unamplified samples with low viral load, in the first round of DNA amplification, the volume of DNA extract used was increased from 10 µl to 20 µl along with reduction in the volume of Milli Q water from 14.5 µl to 4.5 µl. And subsequently in the second round of amplification, the volume of first round product used was increased from 5 µl to 10 µl with reduction in the volume of Milli Q water used to 14.5 µl.

4.2.5 HIV-1 *pol* gene sequencing for Plasma, DBS and PBMC:

Pre-sequencing clean up

The amplified products contained in their original amplification tubes were then purified with Millipore (Billerica, MA, USA) plates by vacuum drying. The pre-sequencing clean up steps were as follows:

1) The amplified product was made up to 100 µl using sterile distilled water.
2) The diluted product was transferred to a Millipore microtiter plate and attached to a Millipore vacuum manifold.
3) Vacuum pressure was applied for approximately 15 minutes or until the well dried.
4) On drying of the wells, 100 µl of sterile distilled water was added and Step 3 was repeated.
5) On complete drying of the wells, 20 µl of sterile distilled water was added to the plate and mixed for 2 minutes on a mechanical shaker.

6) The purified DNA was pipetted from the wells of the microtiter plate and transferred to PCR tubes which were then subjected to sequencing using the Big Dye terminator assay.

Sequencing PCR

The sequencing is performed in our institution using 3 forward (A, B and C) and 3 reverse (E, F and G) in-house sequencing primers as shown in **Table 16**, which are freshly reconstituted to a concentration of 1 picomole/ µl for each use.

Table 16: Sequencing primer sequences

Forward primer	**Sequence**
A primer	AGCCAACAGCCCCACCAG
B primer	GTTAAACAATGGCCATTGACAGAAGA
C primer	TGGAAAGGATCACCAGCAATATTCCA
Reverse primer	**Sequence**
E primer	GGGCCATCCATTCCTGGC
F primer	CCATCCCTGTGGAAGCACATTG
G primer	CTGTATTTCTGCTATTAAGTCTTTTGATG

The sequencing also uses Ready Reaction buffer (ABI PRISM Big Dye terminator cycle sequencing reagent, Applied Biosystems, California, USA) and Ready Reaction mix (ABI PRISM Big Dye terminator cycle sequencing reagent, Applied Biosystems, California, USA). The reaction mix and the cycling conditions for sequencing are shown in **Tables 17** and **18**, respectively.

Table 17: Reaction mix for sequencing

Ingredients	For 1 reaction (µl)
Ready Reaction buffer	2
Ready Reaction mix	1
Freshly reconstituted primer	3.2
Milli Q water	2.8
Purified product	1
Total volume	**10 µl**

Table 18: Cycling conditions for sequencing

Cycle temperature	Cycle time	Number of cycles
96°C	15 minutes	25
50°C	20 seconds	25
60°C	4 minutes	25
15°C	30 minutes	1

The sequencing PCR was carried out in thermal cyclers, Veriti (Applied Biosystems, California, USA) or GeneAmp (Applied Biosystems, Foster City, California, USA).

Post-sequencing clean up

The sequenced samples were then subjected to post-sequencing clean up using a Millipore vacuum system. The steps of purification were as follows:

1) The sequenced sample product was made up to 40 µl using injection solution.
2) The diluted product was transferred to the Millipore microtiter plate and attached to a Millipore vacuum manifold.
3) Vacuum pressure was applied for approximately 10 minutes or until the well dried.

4) On drying of the wells 40 µl of injection solution was added and Step 3 was repeated.

5) On complete drying of the wells, 30 µl of injection solution was added and mixed for 2 minutes on a mechanical shaker and the products were transferred to PCR tubes for analysis.

Sequence analysis

The sequenced products were analysed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The output was in the form of an electropherogram. The complete protease gene was sequenced whereas only the first 328 codons of RT were sequenced. Consensus sequence was created using BioEdit sequence alignment editor version 7.09.0, a representative image is shown in **Figure 12**.

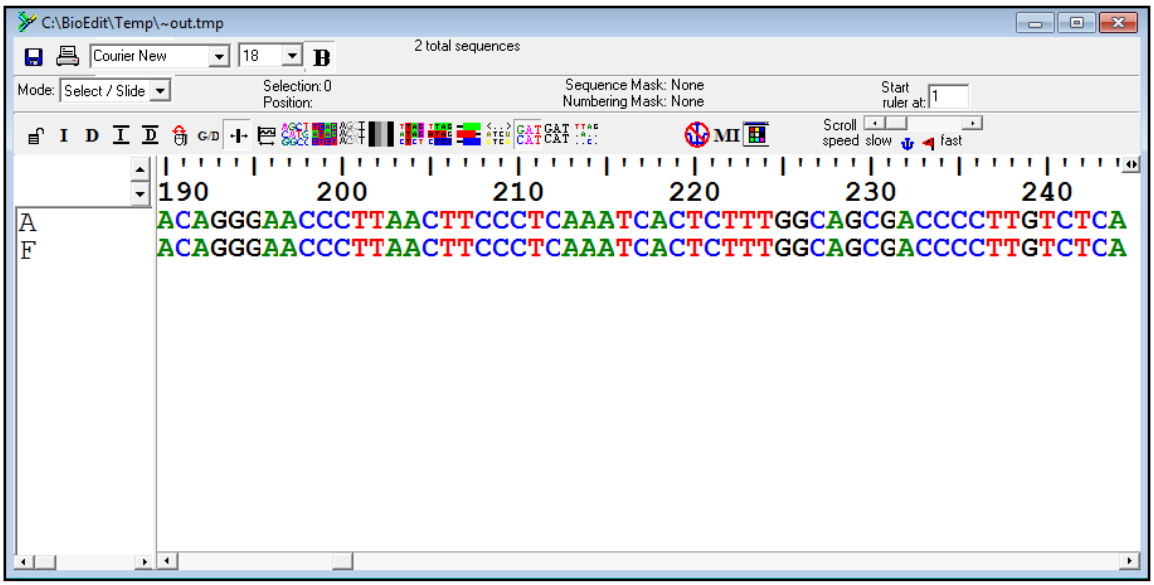


Figure 12: Consensus created on BioEdit sequence alignment editor

The sequences were analysed using Stanford HIV drug resistance database (http://hivdb.stanford.edu), where each mutation was scored against each ARV and based on the sum of the scores, the drug was classified into susceptible (Score: 0 – 9), potentially low-

level resistance (10 – 14), low-level resistance (15 – 29), intermediate resistance (30 – 59) and high-level resistance (≥ 60) a representative image is shown in **Figure 13**.

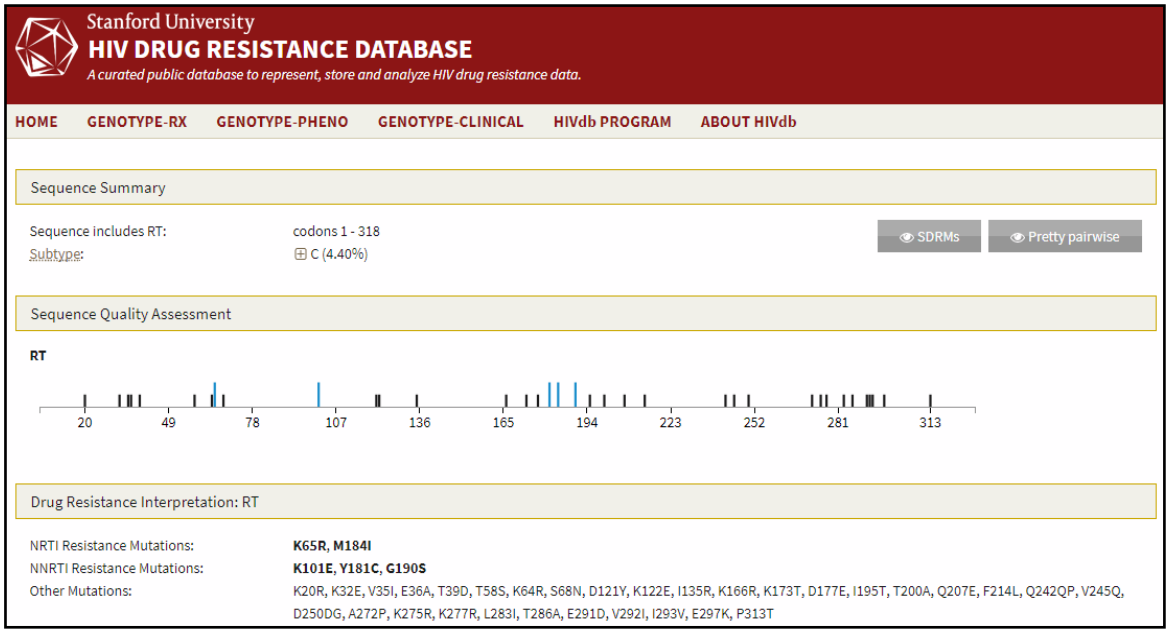


Figure 13: Interpretation of a primer sequence in Stanford HIV drug resistance database

4.2.6 Next Generation Sequencing:

Next generation sequencing was performed on the pre-sequencing clean up products of corresponding plasma, DBS and PBMC from one blood sample which was randomly selected to look for minority variant population.

Ion torrent library preparation

Library preparation was carried out using the Ion Xpress™ Fragment Library Kit, with 100-200 ng of DNA. End repair, adapter ligation, size selection, nick repair and amplification was performed as described in the Ion Torrent protocol associated with the kit.

Qubit 3.0 Fluorometer was used to determine the concentrations of the libraries. The amount of library required for template preparation was calculated using the Template Dilution Factor calculation described in the protocol.

Emulsion PCR and enrichment steps was carried out using the Ion Xpress™ Template Kit and associated protocol. Assessment of the Ion Sphere Particle quality was undertaken between the emulsion PCR and enrichment steps.

Ion torrent sequencing

Sequencing was done using 316 chips in all cases and barcoding was used for these samples. The Ion Sequencing Kit v2.0 was used for all sequencing reactions, following the recommended protocol and Torrent Suite 4.4 was used for all analyses.

The sequences obtained from NGS was compared to the existing DNA libraries available in the NCBI database using BLAST.

4.3 Statistical Analysis

The Mean with Standard Deviation or Median with Interquartile Range was calculated for the continuous variables. Frequencies with percentages were calculated for categorical variables. Sensitivity was calculated for each test with plasma as the Gold Standard test. The comparison of sensitivity across the two tests was done using normal test for proportion. Interclass Correlation Coefficient was calculated for comparing the number of mutations across the two tests. P value < 0.05 was considered as statistical significance. SPSS Statistics version 16.0 was used for analysis.

5. Results

Demography of the study participants

During the study period – July 2016 to August 2017 – 36 individuals were referred to Department of Clinical Virology for genotypic drug resistance testing from plasma. Out of the 36 individuals, 3 were missed due to miscommunication. From the remaining 33 individuals, 1 individual was treatment naïve and was hence excluded as per the exclusion criteria. Of the remaining 32 samples from individuals, 4 samples had undetectable plasma viral loads. So the remaining 28 individuals were included in the study.

Since the required sample size of 34 was not attainable, 4 samples of individuals who were on ART referred for HIV-1 plasma viral load testing were also collected and one of them with detectable plasma viral load was included in the study, thus making a total of 29 samples. An informed consent was taken from all participants at the time of sample collection. The month-wise distribution of the individuals recruited is shown in **Figure 14**.

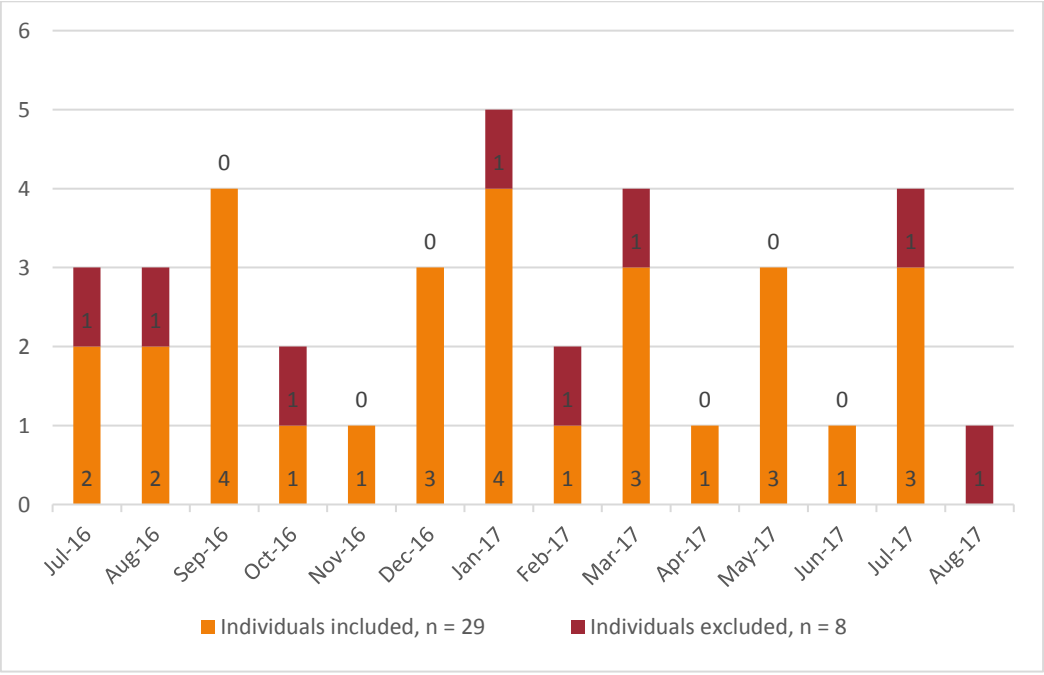


Figure 14: Month-wise distribution of individuals (n = 29) who were referred for testing and the number of individuals who were recruited or excluded.

The 29 individuals who were recruited for the study, hailed from different states of the country. The majority of them were from Tamil Nadu (n = 13, 44.83%) and Andhra Pradesh (n = 12, 41.38%). The geographic distribution of the individuals included in the study are shown in the **Table 19**.

Table 19: The state of origin of the individuals (n = 29) in the study

State	Number (%)
Tamil Nadu	13 (44.83%)
Andhra Pradesh	12 (41.38%)
Kerala	2 (6.89%)
Bihar	1 (3.45%)
Jharkhand	1 (3.45%)
Total	**29 (100%)**

Among the individuals who were recruited for the study, male = 23 and female = 6. The gender distribution is represented in **Figure 15**.

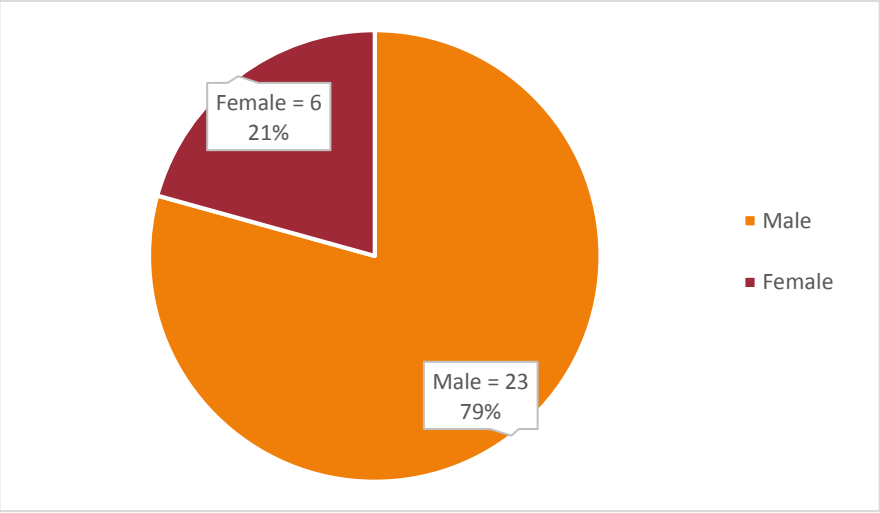


Figure 15: The gender ratio of the individuals (n = 29) in the study.

The age distribution of the individuals recruited for the study ranged from 18 to 56 years, as shown in **Table 20**. And the mean age was 44.2 ± 8.49 years (Males = 44.52 years; Females = 43 years) and the median age was 46 (Interquartile range: 38 - 50) years, (Males = 47 years; Females = 44.5 years).

Table 20: The age groups of the individuals in the study (n = 29)

Age group in years	Number (%)
18 – 30	1 (3.45%)
31 – 40	9 (31.03%)
41 – 50	12 (41.38%)
51 – 60	7 (24.14%)
Total	**29 (100%)**

CD4 T cell count of the study participants

The CD4 cell count – the indicator of immunological failure – of the individuals in the study at the time or closest to that of sample collection, ranged between 5 and 775 cells/ μl. The mean and the median CD4 counts were 209 ± 190 cells/ μl and 155 (Interquartile range: 60 – 325) cells/ μl, respectively. The study participants grouped together based on their CD4 cell count have been represented in **Table 21.**

Table 21: The distribution of individuals in the study based on their CD4 counts.

CD4 cell counts in cells/ μl	Number (%)
< 100	11 (37.9%)
100 – 200	6 (20.7%)
201 – 300	2 (6.9%)
301 – 400	6 (20.7%)
401 – 500	2 (6.9%)
> 500	2 (6.9%)

Plasma viral load levels of the study participants

The plasma viral load (VL) of the individuals in the study at the time or closest to that of sample collection, ranged between 615 to 1,735,708 copies/ ml. The mean and median viral load levels were 254,477 copies/ ml and 58,242 (Interquartile range: 6112 – 195,236) copies/ ml, respectively. The mean and median viral load expressed as log_{10} copies/ ml was log_{10} 4.65 ± 1.03 copies/ ml and log_{10} 4.77 (Interquartile range: 3.74 – 5.34) copies/ ml, respectively. The distribution of the individuals in the study based on their viral load is shown in **Table 22**.

Table 22: The distribution of individuals in the study based on their viral load.

Viral load in log_{10} copies/ ml	Number (%)
< 3	3 (10.34%)
3 – 3.99	5 (17.24%)
4 – 4.99	8 (27.59%)
5 – 5.99	11 (37.93%)
6 – 6.99	2 (6.9%)

Duration of treatment of the study participants

The duration of taking ART was known for 28 of the individuals in the study and ranged from 3 months to 16 years, while the treatment duration for 1 individual was not known. The mean and median duration of receiving ART was 5.93 ± 4.64 and 5 (Interquartile range: 1.13 - 10) years. The distribution of the study participants based on the duration of receiving treatment is shown in **Table 23**.

Table 23: The distribution of the individuals in the study based on the duration of treatment

Duration of ART in years	Number (%)
≤ 2	9 (32.1%)
2.1 - 6	7 (25%)
6.1 - 10	8 (28.6%)
> 10	4 (14.3%)
Total	**28 (100%)**

ART regimens

The ART received by 28 participants in the study was known but the regimen followed by 1 individual was unknown. The different ART regimens followed by the study participants at the time of sample collection are shown in **Figure 16**.

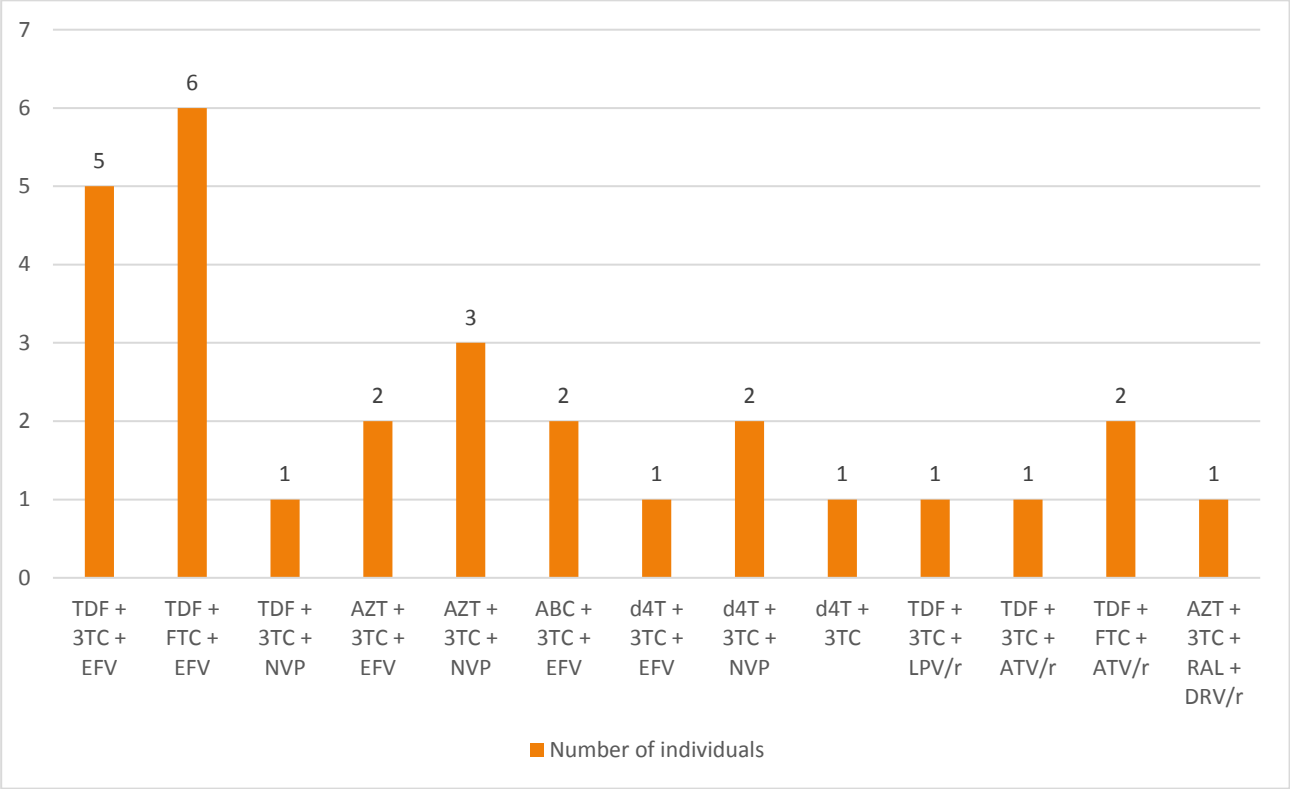


Figure 16: The different ART regimens followed by the individuals in the study at the time of sample collection. (TDF = Tenofovir, 3TC = Lamivudine, EFV = Efavirenz, FTC = Emtricitabine, NVP = Nevirapine, AZT = Zidovudine, ABC = Abacavir, d4T = Stavudine, LPV/r = ritonavir boosted Lopinavir, ATV/r = ritonavir boosted Atazanavir, DRV/r = ritonavir boosted Darunavir, RAL = Raltegravir)

Of these 28 individuals, 13 never switched their ART regimen whereas 10 switched their therapy once, 4 of them switched twice and 1 individual had 4 switches in regimen, as represented in **Table 24**.

Table 24: Treatment switches among study participants

Treatment regimen	Number (%)
Never switched	13 (46.4%)
1 switch in regimen	10 (35.7%)
2 switches in regimen	4 (14.3%)
3 switches in regimen	0
4 switches in regimen	1 (3.6%)

Among the 28 individuals whose regimen was known, 6 individuals had discontinued their treatment and then restarted it and 2 were highly irregular or poorly adherent to the therapy.

Opportunistic infections and HIV associated manifestations

Based on the history from the participants and the clinical findings, diagnosis and laboratory reports obtained from the patient charts, it was observed that of all the 29 individuals included in the study, 26 (89.7%) of them had suffered from various opportunistic infections or HIV associated illness during the course of the disease.

The different opportunistic infections seen among the participants were cutaneous fungal infections, oral candidiasis, herpes zoster, herpes genitalis, tuberculosis (pulmonary, extra-pulmonary or disseminated), *Pneumocystis jirovecii* pneumoniae, CMV retinitis, progressive multifocal leukoencephalopathy, cryptococcal meningitis, cryptococcemia and isosporiasis. The other manifestations seen were HIV-associated cognitive decline or neurocognitive disorders.

WHO Clinical Staging

Depending on the clinical manifestations seen during the course of their illness, the 29 individuals in the study were classified by the attending clinicians into WHO Clinical Stages 1 – 4. This data was obtained from the patient records. The distribution of study subjects based on the WHO Staging is represented in **Figure 17**.

The 3 asymptomatic subjects were classified into Stage 1 and their CD4 counts ranged from 330 – 579 cells/ µl. Four individuals who had herpes zoster were classified into Stage 2 with CD4 counts ranging from 37 – 319 cells/ µl. Three individuals with CD4 counts 75 – 479 cells/ µl were categorised into Stage 3 and had pulmonary tuberculosis and oral candidiasis. The remaining 19 subjects were grouped under Stage 4 and had varied manifestations like extra-pulmonary tuberculosis (TB lymphadenitis), disseminated tuberculosis, *Pneumocystis* pneumonia, CMV retinitis, progressive multifocal leukoencephalopathy, cryptococcal meningitis, cryptococcemia, isosporiasis and HIV associated neurocognitive disorders. And the CD4 counts were in the range of 5 – 357 cells/ µl.

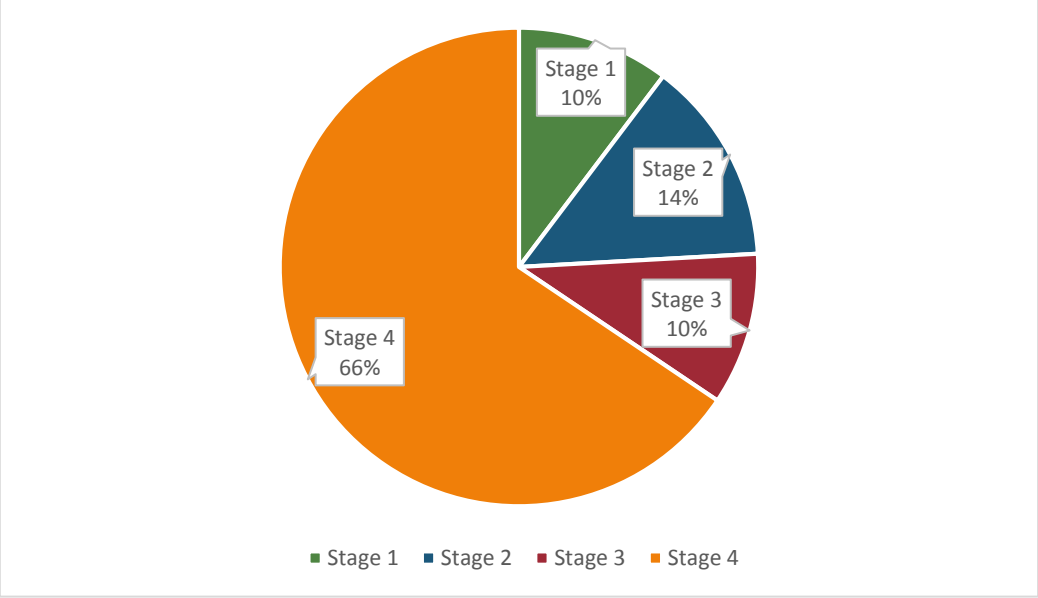


Figure 17: The proportion of individuals in the study belonging to different WHO Clinical Stages.

pol gene Amplification

All 3 components (plasma, DBS and PBMC) of each collected blood sample was subjected to a nested PCR to amplify the *pol* gene of HIV-1. The amplified products were run on a 2% agarose gel to check for the specific 1800 base pair size amplicons and a representative gel documentation picture is shown in **Figure 18**.

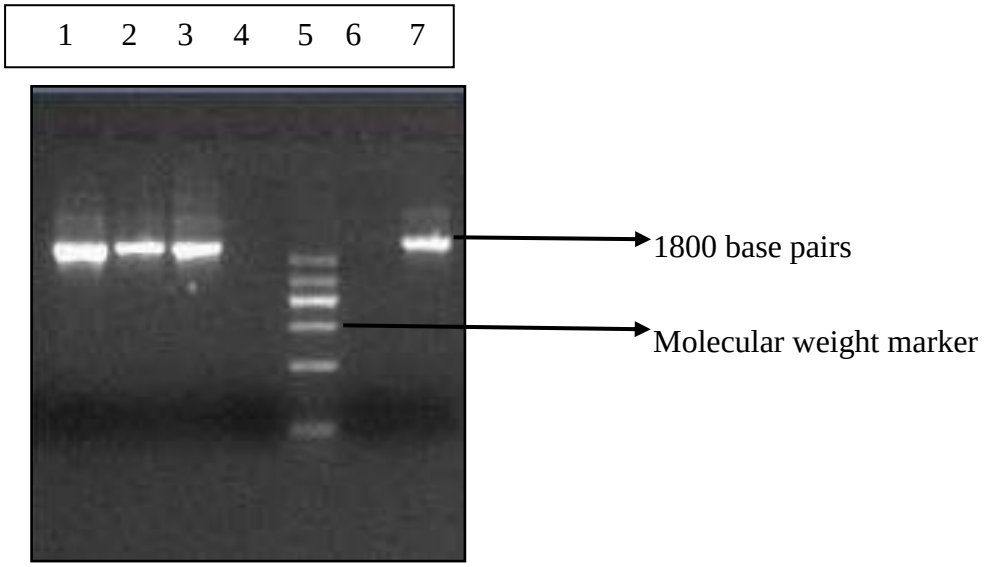


Figure 18: Representative gel documentation picture of amplification of *pol* gene. (Lanes 1, 2 and 3: Positive samples; Lane 4: Negative sample; Lane 5: Molecular ladder; Lane 6: Negative control; Lane 7: Positive control)

Out of the 29 samples collected, 26 (89.7%) had a viral load > 1000 copies/ ml while remaining 3 (10.3%) had < 1000 copies/ ml.

From the 26 samples with viral load > 1000 copies/ ml, 25 (96.2%) amplified from the gold standard for HIV-1 genotyping, plasma sample. Whereas from DBS and PBMC, 19 (73.1%) and 26 (100%) samples amplified, respectively. The amplification success for each sample type when the VL > 1000 copies/ ml is represented in **Figure 19**.

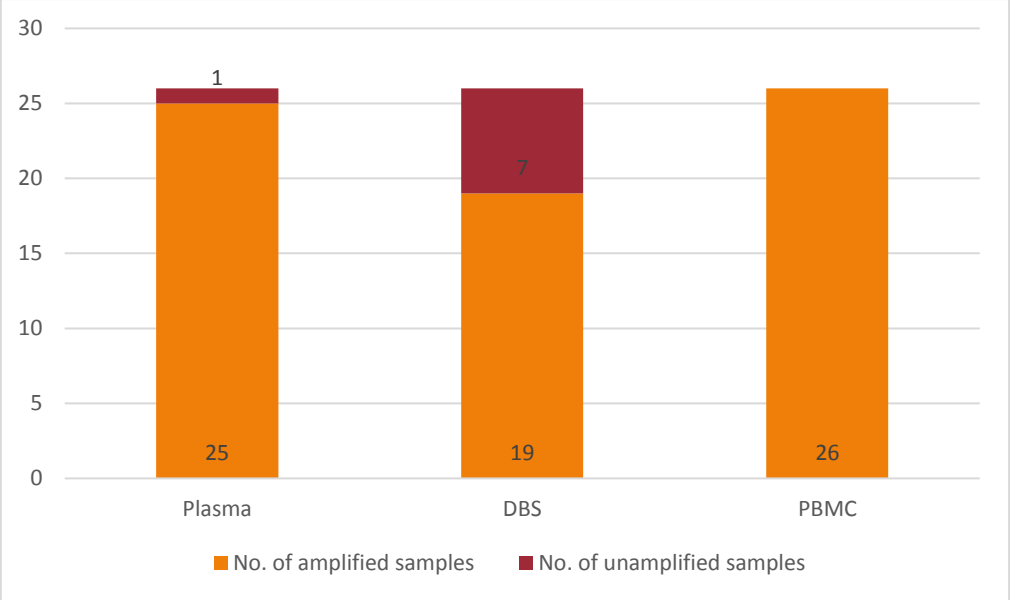


Figure 19: The amplification success among the three sample types with viral load > 1000 copies/ml.

The viral load of the amplified samples ranged from 3.02 – 6.24 $\log_{10}$ copies/ ml. And the viral load of the samples that did not amplify in plasma and DBS are listed in **Table 25**.

Table 25: The viral loads of the unamplified samples

Sample Identification No.	Viral load in $\log_{10}$ copies/ ml
Plasma DR 16/38	3.4
DBS-C-011	3.38
DBS-C-023	3.53
DBS-C-014	4.98
DBS-C-018	5.14
DBS-C-022	5.27
DBS-C-021	5.39
DBS-C-005	5.79

Among the remaining 3 samples with viral load < 1000 copies/ ml, 1 (33.3%) amplified from plasma, 1 (33.3%) from DBS and all 3 (100%) from PBMC. The amplification success for each sample type when the VL < 1000 copies/ ml is represented in **Figure 20**.

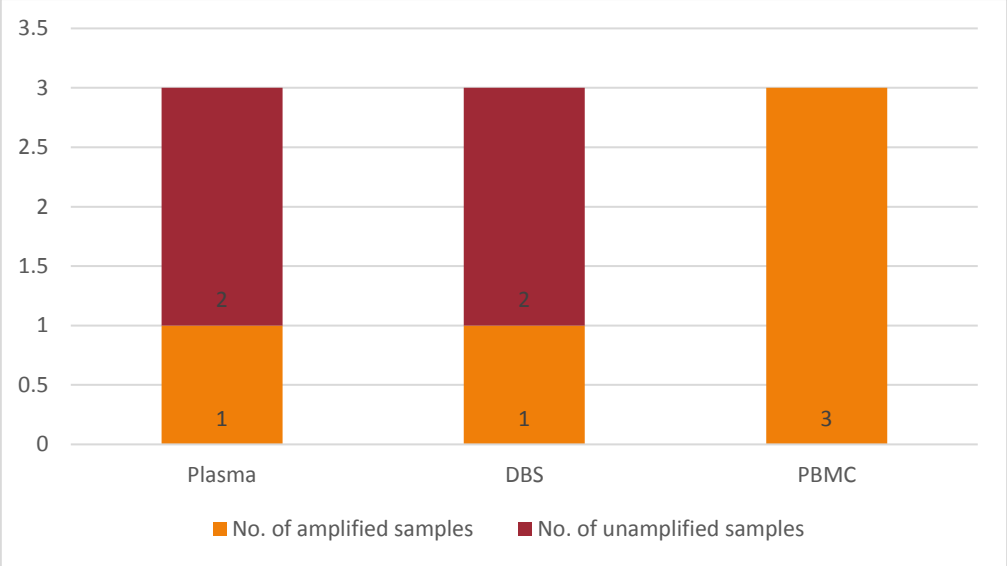


Figure 20: The amplification success among the three sample types with viral load < 1000 copies/ml.

It was observed that one sample did not amplify both in plasma and DBS. The viral load of the unamplified samples are listed in **Table 26**.

Table 26: The viral load of the unamplified samples

Sample Identification No.	**Viral load in log_{10} copies/ ml**
Plasma DR 17/08 & DBS-C-019	2.79
Plasma DR 16/42	2.88
DBS-C-020	2.94

Meanwhile all the PBMC samples amplified (100%), including the one with the lowest viral load of 2.79 log_{10} copies/ ml, which did not amplify in plasma and DBS.

Totally, 26 samples amplified in plasma out of the 29 included in the study and 8 of these did not amplify in DBS, as represented in **Figure 21**.

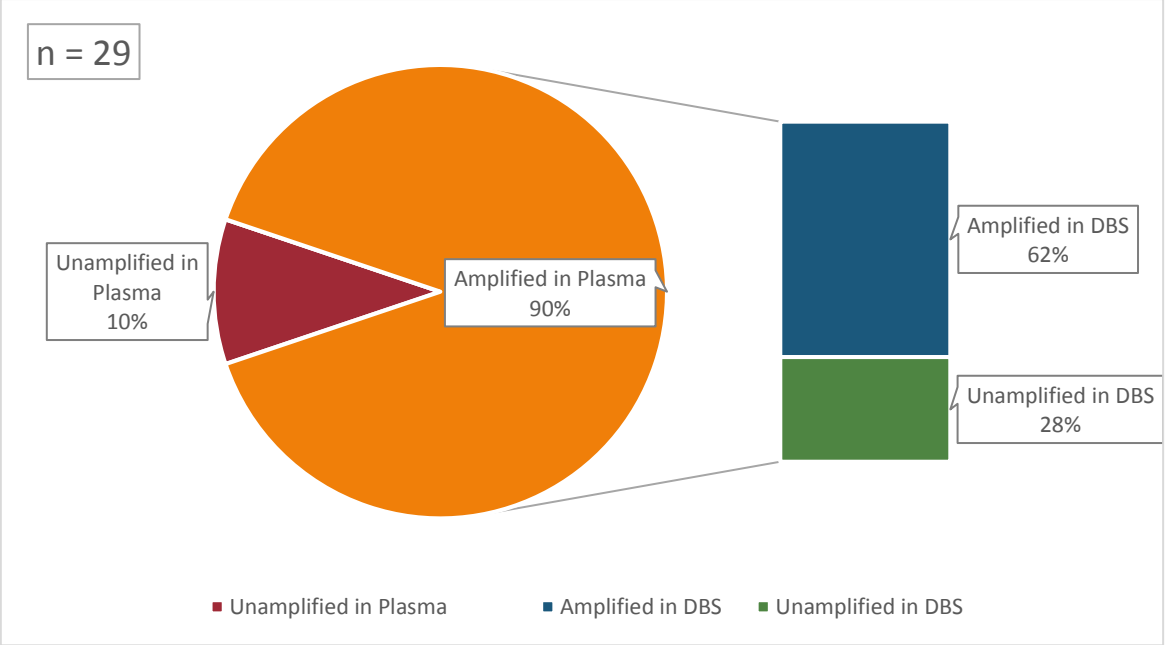


Figure 21: From the total (n = 29), the proportion of samples that amplified and did not amplify in DBS among those that amplified in plasma.

Totally 20 samples amplified in DBS out of the 29 included in the study and 2 of these did not amplify in plasma as represented in **Figure 22**.

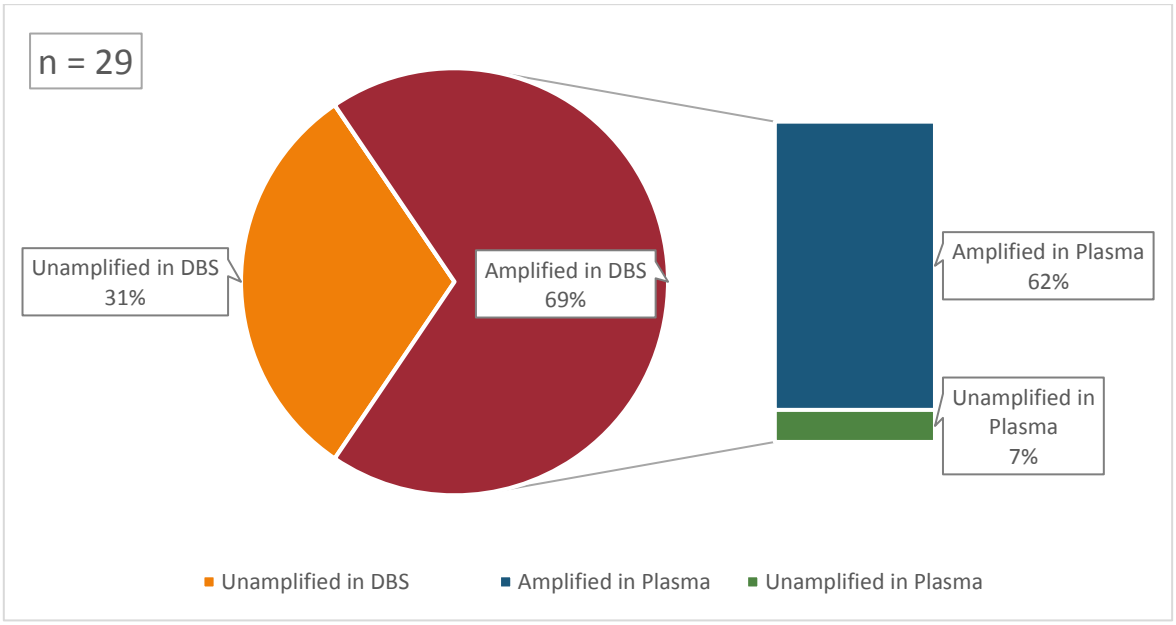


Figure 22: From the total (n = 29), the proportion of samples that amplified and did not amplify in plasma among those that amplified in DBS.

Of the total 29 samples, between plasma, DBS and PBMC, 18 samples amplified in all three sample types. Ten samples were sucessfully amplified in any 2 sample types and one sample amplified only from one sample type, as represented in **Figure 23**.

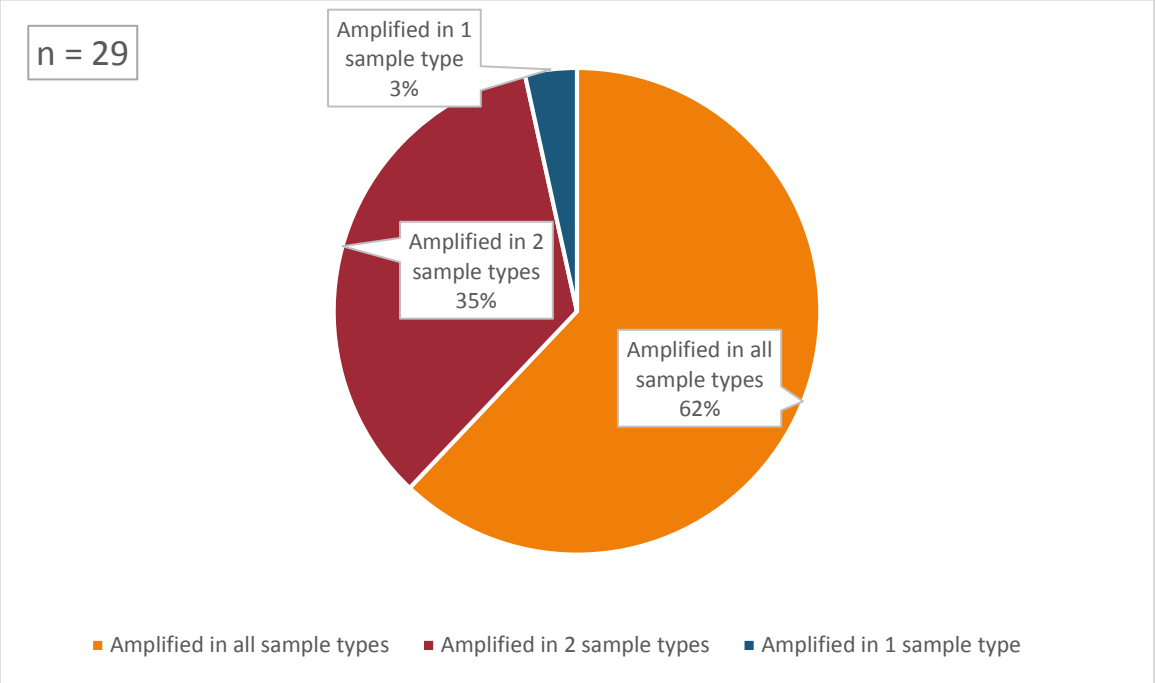


Figure 23: Amplification success rate of the total samples (n = 29).

The overall sensitivity of DBS and PBMC with respect to plasma for 29 samples was 69.2% and 100%, respectively. While taking plasma as the gold standard, the difference between the sensitivities of DBS and PBMC was found to be significant, p = 0.0021.

Sequencing

All amplified samples were sequenced by conventional Sanger's sequencing and the resultant electropherograms were analysed using Stanford HIV drug resistance database. A representative electropherogram obtained from one of the sequence primers is shown in **Figure 24**.

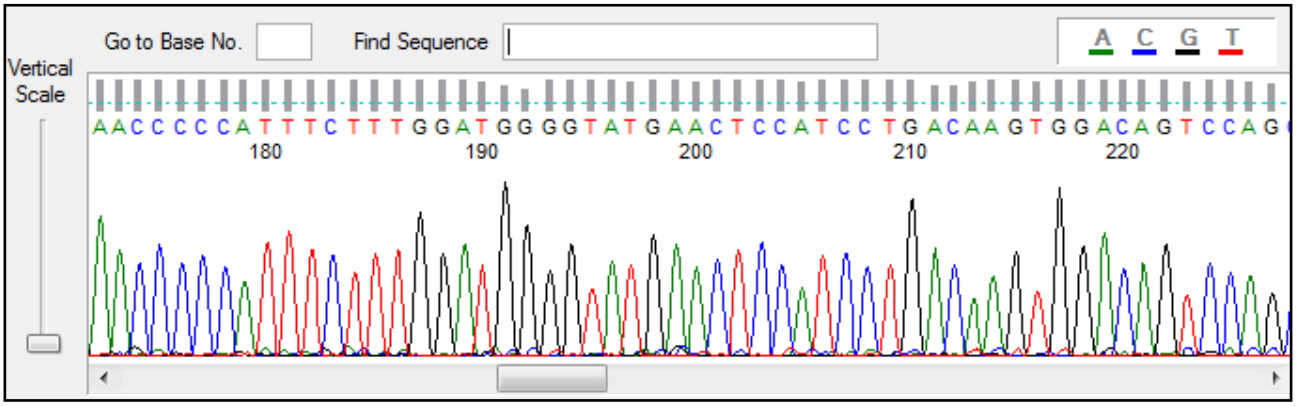


Figure 24: Representative electropherogram of the sequences.

There could be slight differences in mutations between 2 sample types but the drug resistance profile could remain unchanged since all mutations do not lead to clinically significant drug resistance. For the analysis of all samples, concordance was defined as identical drug resistance profile between any pair of sequences from a individual's plasma and DBS or PBMC. And discordance as discordant drug resistance profiles.

When the sequences from the 26 samples which amplified in plasma were compared with corresponding PBMC sequences, 20 (76.9%) were found to be concordant. Among them, 14 were found to have identical drug resistance mutations and 4 did not have any drug resistance mutations. Although the remaining two had concordant resistance profiles, identified mutations were slightly different between the two compartments. The details and summary of mutations detected in the concordant samples are listed in **Tables 27**, **28** and **29**.

Table 27: Samples with no drug resistant mutations detected in plasma and PBMC

Sample No.	Viral load in log_{10} copies/ml	Duration of treatment	Latest ART regimen	Treatment switches
DBS-007	4.15	1 year	Tenofovir + Emtricitabine + Efavirenz	Nil
DBS-012	4.77	10 years	Abacavir + Lamivudine + Efavirenz	1 switch
DBS-013	6.22	10 years	Tenofovir + Emtricitabine + Efavirenz	Nil (poor adherence)
DBS-033	5.29	Unknown	Unknown	Unknown

Among the 14 samples with identical mutations in plasma and PBMC, only 1 had mutations that conferred resistance to protease inhibitors and that individual was taking Tenofovir, Emtricitabine and ritonavir boosted Atazanavir. All 14 had mutations that conferred resistance to non-nucleoside reverse transcriptase inhibitors and all except one had mutations against nucleotide reverse transcriptase inhibitors.

Table 28: Samples with identical drug resistance mutations

Sample No.	PI mutations	NRTI resistance mutations	NNRTI resistance mutations
DBS-001	None	D67G, K70E, M184V	V106M, Y188L
DBS-002	None	M41L, L74I, M184V, T215Y	A98G, K101E, V108I, E138A, G190S
DBS-004	None	M41L, M184V, T215Y	A98G, K103N, K238T
DBS-005	None	K65R, M184V, K219Q	L100I, K103N, P225H
DBS-006	None	M184V	K103N, V106M
DBS-014	None	D67N, K70R, M184V, T215I, K219E	V179E, G190A, P225H
DBS-016	None	None	K103KN, G190A
DBS-018	None	D67N, K70R, M184V, T215I, K219E	V106M, G190A, F227L
DBS-021	None	D67G, K70E	K103N, V106M
DBS-023	K20T, Q58E, G73S	M41L, E44D, D67N, K70R, V75M, M184V, T215Y, K219E	A98G, K101E, V108I, Y181C, G190A
DBS-025	None	D67N, K70R, M184V, T215I, K219Q	Y181C
DBS-026	None	K70E, M184V	K103N, V108I, H221Y
DBS-030	None	M184V	V106M
DBS-032	None	M41L,K65R,Y115F, M184V	V106M, V179D

(PI = Protease inhibitors, NRTI = Nucleoside reverse transcriptase inhibitors, NNRTI = Non-nucleoside reverse transcriptase inhibitors)

The 2 samples with additional mutations in sequences obtained from plasma or PBMC without any difference in drug resistance profiles were also considered as concordant.

Table 29: Samples with additional mutations but identical resistance profiles. (Additionally detected mutations are highlighted)

Sample No.	Mutations in PR	Mutations in RT
Plasma DR 17/05	None	K65R, M184I, K101E, Y181C, G190S, **H221Y**
DBS-011	None	M184V, K103N, Y188YF, H221HY, **Y318YF, N348I**

(PR = Protease, RT = Reverse transcriptase)

Of the 26 amplified samples from plasma, 6 were discordant with PBMC (**Table 30** and **31**). Discordance was due mutations being identified only in plasma or only in PBMC which altered the resistance profiles.

Table 30: Mutations detected in specimens with discordant drug resistance profiles between plasma RNA and PBMC DNA (Additionally identified mutations are highlighted)

Sample No.	Mutations in plasma		Mutations in PBMC	
	PR	**RT**	**PR**	**RT**
DBS-003	None	**M41L, D67N, K70R, L74I, M184V, T215F, K219Q, V106M, G190A, F227L, M230L**	None	None
DBS-009	**V32I, M46I, I54M, I84V, L90M, L33F,** G73S, **L89V**	M41L, D67N, K70R, M184V, T215F, **K219E,** A98G, Y181C	G73S	M41L, D67N, K70R, M184V, T215F, A98G, Y181C
DBS-015	None	M41L, **E44D, D67N, V75M,** M184V, **L210W**, T215Y, K103N, **V108I,** Y318F	None	M41L, M184V, T215TNSY, K103KN, Y318F
DBS-020	**I54V, L90M, K20T**	**D67N, K70R, M184V, T215Y, K219E,** K103N, P225H	None	K103N, P225H
DBS-022	**V82A**	D67N, T69D, K70R, M184V, K219E, L100I, K103N	None	D67N, T69D, K70R, M184V, K219E,L100 I, K103N, N348I
DBS-024	None	M184I, Y181C	None	M184I, **K219N,** Y181C

(PR = Protease, RT = Reverse transcriptase)

Table 31: The altered drug resistance profile of discordant samples. (The difference in resistance profile for each drug is highlighted and underlined. PL = Plasma, PBM = PBMC, S = Susceptible, PLR = Potential low-level resistance, LR = Low-level resistance, IR = Intermediate resistance, HR = High-level resistance)

Drugs	DBS-003		DBS-009		DBS-015		DBS-020		DBS-022		DBS-024	
	PL	PBM	PL	PBM	PL	PBM	PL	PBM	PL	PBM	PL	PBM
ATV/r	S	S	**HR**	S	S	S	**IR**	S	**LR**	S	S	S
DRV/r	S	S	**HR**	S	S	S	S	S	S	S	S	S
FPV/r	S	S	**HR**	S	S	S	**IR**	S	**LR**	S	S	S
IDV/r	S	S	**HR**	S	S	S	**HR**	S	**IR**	S	S	S
LPV/r	S	S	**HR**	S	S	S	**IR**	S	**IR**	S	S	S
NFV	S	S	**HR**	S	S	S	**HR**	S	**IR**	S	S	S
SQV/r	S	S	**HR**	S	S	S	**HR**	S	**LR**	S	S	S
TPV/r	S	S	**HR**	S	S	S	**LR**	S	S	S	S	S
ABC	**HR**	S	**HR**	IR	**HR**	IR	**HR**	S	HR	HR	LR	LR
AZT	**HR**	S	**HR**	IR	**HR**	IR	**HR**	S	IR	IR	S	S
d4T	**HR**	S	**HR**	IR	**HR**	IR	**HR**	S	IR	IR	S	S
DDI	**HR**	S	**HR**	IR	**HR**	IR	**HR**	S	HR	HR	**PLR**	LR
FTC	**HR**	S	HR	HR	HR	HR	**HR**	S	HR	HR	HR	HR
3TC	**HR**	S	HR	HR	HR	HR	**HR**	S	HR	HR	HR	HR
TDF	**HR**	S	**IR**	LR	**HR**	LR	**IR**	S	LR	LR	S	S
EFV	**HR**	S	IR	IR	HR	HR	HR	HR	HR	HR	IR	IR
ETR	**IR**	S	IR	IR	S	S	S	S	IR	IR	IR	IR
NVP	**HR**	S	HR	HR	HR	HR	HR	HR	HR	HR	HR	HR
RPV	**HR**	S	HR	HR	S	S	S	S	HR	HR	IR	IR

(ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, SQV/r, TPV/r = ritonavir boosted Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Saquinavir and Tipranavir respectively, NFV = Nelfinavir,

ABC = Abacavir, AZT = Zidovudine, d4T = Stavudine, DDI = Didanosine, FTC = Emtricitabine, 3TC = Lamivudine, TDF = Tenofovir, EFV = Efavirenz, ETR = Etravirine, NVP = Nevirapine, RPV = Rilpivirine)

Out of the total 29 samples, 3 which did not amplify in plasma but amplified in PBMC showed the following mutations and drug resistance profile as listed in **Table 32**. Two of these samples had mutations which impacted the resistance profile which would necessitate a switch in the ART regimen followed by the individual. And no mutations were detected in the third sample.

Table 32: Mutations identified and drug resistance profile of 3 samples which amplified in PBMC and not plasma (S = Susceptible, PLR = Potential low-level resistance, LR = Low-level resistance, IR = Intermediate resistance, HR = High-level resistance)

Sample No.	**Mutations detected**		**Resistance profile**
	PR	**RT**	
DBS-008	None	D67Deletion, T69G, K70R, M184V, K219Q, K101H, K103N, G190A, Y318F	**S =** ATV/r, DRV/r, LPV/r, FPV/r, IDV/r, NFV SQV/r, TPV/r **LR =** ETR, RPV **IR =** d4T, TDF **HR =** ABC, AZT, DDI FTC, 3TC, EFV, NVP
DBS-010	None	M184V, Y181C	**S =** ATV/r, DRV/r, LPV/r, FPV/r, IDV/r, NFV, SQV/r, TPV/r, AZT, d4T, TDF **PLR =** DDI **LR =** ABC **IR =** EFV, ETR, RPV **HR =** FTC, 3TC, NVP
DBS-019	None	None	**S =** All

(ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, SQV/r, TPV/r = ritonavir boosted Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Saquinavir and Tipranavir respectively, NFV = Nelfinavir, ABC = Abacavir, AZT = Zidovudine, d4T = Stavudine, DDI = Didanosine, FTC = Emtricitabine, 3TC = Lamivudine, TDF = Tenofovir, EFV = Efavirenz, ETR = Etravirine, NVP = Nevirapine, RPV = Rilpivirine) (PR = Protease, RT = Reverse transcriptase)

When sequences from the 18 samples which amplified in DBS and plasma were compared, 10 were found to be concordant. Among them, 7 were found to have identical drug resistance mutations and 2 did not have any drug resistance mutations. Although the remaining one had concordant resistance profiles, identified mutations were slightly different between the two compartments.

Samples DBS-013 and DBS-033 did not have any drug resistance mutations in plasma, DBS and PBMC. The treatment details of these two individuals are already listed in **Table 27**.

The details and summary of mutations detected in the concordant samples are listed in **Tables 33** and **34**.

Among the 7 samples with identical mutations in plasma and DBS, none had mutations that conferred resistance to protease inhibitors. All 7 had mutations that conferred resistance to non-nucleoside reverse transcriptase inhibitors and all except one had mutations against nucleotide reverse transcriptase inhibitors.

Table 33: Samples with identical drug resistance mutations

Sample No.	PI mutations	NRTI mutations	NNRTI mutations
DBS-017	None	K65R, M184I	K101E, Y181C, G190S, H221Y
DBS-024	None	M184I	Y181C

(Samples DBS-001, DBS-006, DBS-016, DBS-030 and DBS-032 are the other concordant samples and are listed in **Table 28.** PI = Protease inhibitors, NRTI = Nucleoside reverse transcriptase inhibitors, NNRTI = Non-nucleoside reverse transcriptase inhibitors)

One sample with additional mutations in sequences obtained from DBS without any difference in drug resistance profiles was also considered as concordant.

Table 34: Sample with additional mutations but identical resistance profiles. (Additionally identified mutations are highlighted)

Additional mutations identified only in	Mutations in PR	Mutations in RT
DBS-009	V32I, M46I, I54M, **V82A**, I84V, L90M, L33F, G73S, L89V	M41L, D67N, K70R, M184V, T215F, K219E, A98G, Y181C

(PR = Protease, RT = Reverse transcriptase)

Of the amplified 18 samples from plasma and DBS, 8 were discordant. Discordance was due mutations being identified only in plasma or only in DBS which altered the drug resistance profiles. The specimens with discordant mutations are listed in **Table 35**.

Table 35: Specimens with discordant drug resistance profiles between plasma and DBS (Additionally identified mutations are highlighted) (PR = Protease, RT = Reverse transcriptase)

Sample No.	Mutations in plasma		Mutations in DBS	
	PR	**RT**	**PR**	**RT**
DBS-002	None	M41L, L74I, M184V, T215Y, A98G, K101E, V108I, E138A, G190S	None	M41L, **K70E,** M184V, T215Y, L74I, A98G, K101E, E138A, G190C, V108I
DBS-003	None	**M41L, D67N, K70R, L74I, M184V, T215F, K219Q, V106M, G190A, F227L, M230L**	None	None
DBS-004	None	M41L, M184V, T215Y, A98G, **K103N,** K238T	None	M41L, M184V, T215Y, A98G, K238T
DBS-007	None	None	None	**D67N, V106M, G190E**
DBS-012	None	None	None	**K65R, V106M**
DBS-015	None	M41L, **E44D, D67N,V75M,** M184V, **L210W,** T215Y, K103N, **V108I,** Y318F	None	M41L, M184V, T215Y, K103N, Y318F
DBS-025	None	D67N, K70R, M184V, **T215I,** K219Q, Y181C	None	D67N, K70R, M184V, K219Q, Y181C
DBS-026	None	K70E, M184V, K103N, V108I, H221HY	None	K70E, M184V, K103N, V108I, **E138G,** H221Y

The discordant drug resistance profiles are listed in **Tables 36**.

Table 36: The altered drug resistance profile of discordant samples. (The difference in resistance profile for each drug is highlighted and underlined. PL = Plasma, S = Susceptible, PLR = Potential low-level resistance, LR = Low-level resistance, IR = Intermediate resistance, HR = High-level resistance)

Drugs	**DBS-002**		**DBS-004**		**DBS-007**		**DBS-012**		**DBS-015**		**DBS-025**		**DBS-026**	
	PL	**DBS**	**PL**	**DBS**	**PL**	**DBS**	**PL**	**DBS**	**PL**	**DBS**	**PL**	**DBS**	**PL**	**DBS**
ATV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
DRV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
FPV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
IDV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
LPV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
NFV	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SQV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
TPV/r	S	S	S	S	S	S	S	S	S	S	S	S	S	S
ABC	HR	HR	IR	IR	S	S	**S**	IR	**HR**	IR	HR	HR	IR	IR
AZT	IR	IR	IR	IR	**S**	LR	S	S	**HR**	IR	**HR**	IR	S	S
d4T	**IR**	HR	IR	IR	**S**	LR	**S**	HR	**HR**	IR	**HR**	IR	LR	LR
DDI	HR	HR	IR	IR	S	S	**S**	HR	**HR**	IR	IR	IR	LR	LR
FTC	HR	HR	HR	HR	S	S	**S**	IR	HR	HR	HR	HR	HR	HR
3TC	HR	HR	HR	HR	S	S	**S**	IR	HR	HR	HR	HR	HR	HR
TDF	**LR**	IR	LR	LR	S	S	**S**	HR	**HR**	LR	LR	LR	LR	LR
EFV	HR	HR	**HR**	IR	**S**	HR	**S**	HR	HR	HR	IR	IR	HR	HR
ETR	IR	IR	PLR	PLR	**S**	IR	S	S	S	S	IR	IR	**PLR**	LR
NVP	HR	HR	HR	HR	**S**	HR	**S**	HR	HR	HR	HR	HR	HR	HR
RPV	HR	HR	LR	LR	**S**	HR	S	S	S	S	IR	IR	**LR**	IR

(ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, SQV/r, TPV/r = ritonavir boosted Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Saquinavir and Tipranavir respectively, NFV = Nelfinavir, ABC = Abacavir, AZT = Zidovudine, d4T = Stavudine, DDI = Didanosine, FTC = Emtricitabine, 3TC = Lamivudine, TDF = Tenofovir, EFV = Efavirenz, ETR = Etravirine, NVP = Nevirapine, RPV = Rilpivirine)

The difference in the drug resistance profile between plasma and DBS of Sample DBS-003 is identical to that of PBMC and is shown in **Table 31**.

Out of the total 26 samples which amplified in plasma, 8 which did not amplify in DBS showed the following mutations and drug resistance profile as listed in **Table 37**.

Table 37: Mutations identified and drug resistance profile of 8 samples which amplified in plasma and PBMC and not in DBS (S = Susceptible, PLR = Potential low-level resistance, LR = Low-level resistance, IR = Intermediate resistance, HR = High-level resistance)

Sample	**Mutations detected**		**Resistance profile**
No.	**PR**	**RT**	
DBS-005	None	K65R, M184V, K219Q, L00I, K103N, P225H	**S =** ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r, AZT **IR =** TDF, ETR **HR =** ABC, d4T, DDI, FTC, 3TC, EFV, NVP, RPV
DBS-011	None	M184V, K103N, Y188F, H221Y	**S =** ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r, AZT, d4T, TDF **PLR =** DDI, ETR **LR =** ABC **IR =** RPV **HR =** FTC, 3TC, EFV, NVP
DBS-014	None	D67N, K70R, M184V, T215I, K219E,V179E, G190A, P225H	**S =** ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r **LR =** TDF, ETR, RPV **IR =** DDI **HR =** ABC, AZT, d4T, FTC, 3TC, EFV, NVP

DBS-018	None	D67N, K70R, M184V, T215F, K219E,V106M, G190A, F227L	**S** = ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r **PLR** = ETR **LR** = RPV **IR** = TDF **HR** = ABC, AZT, d4T, DDI, FTC, 3TC, EFV, NVP
DBS-020	I54V, L90M, K20T	D67N, K70R, M184V, T215Y, K219E, K103N, P225H	**S** = DRV/r, ETR, RPV, **LR** = TPV/r **IR** = ATV/r, FPV/r, LPV/r, TDF, **HR** = IDV/r, NFV, SQV/r, ABC, AZT, d4T, DDI, FTC, 3TC, EFV, NVP
DBS-021	None	D67G, K70E, K103N, V106M	**S** = ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r, AZT, ETR, RPV **PLR** = FTC, 3TC **LR** = ABC, d4T, DDI, TDF **HR** = EFV, NVP
DBS-022	V82A	D67N, T69D, K70R, M184V, K219E, L100I, K103N	**S** = DRV/r, TPV/r **LR** = ATV/r, FPV/r, SQV/r, TDF **IR** = IDV/r, LPV/r, NFV, AZT, d4T, ETR **HR** = ABC, DDI, FTC, 3TC, EFV, NVP, RPV
DBS-023	K20T, Q58E, G73S	M41L, E44D, D67N, K70R, V75M, M184V, T215Y, K219E, A98G, K101E, V108I, Y181C, G190A	**S** = DRV/r, LPV/r **LR** = ATV/r, FPV/r, IDV/r, SQV/r, TPV/r **IR** = NFV, TDF **HR** = ABC, AZT, d4T, DDI, FTC, 3TC, EFV, ETR, NVP, RPV

(ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, SQV/r, TPV/r = ritonavir boosted Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Saquinavir and Tipranavir respectively, NFV = Nelfinavir, ABC = Abacavir, AZT = Zidovudine, d4T = Stavudine, DDI = Didanosine, FTC = Emtricitabine, 3TC = Lamivudine, TDF = Tenofovir, EFV = Efavirenz, ETR = Etravirine, NVP = Nevirapine, RPV = Rilpivirine)

The two samples which amplified in DBS and not in plasma are listed in **Table 38**. And one sample did not amplify both in plasma and DBS.

Table 38: Mutations identified and drug resistance profile of 2 samples which amplified in DBS and PBMC and not in plasma (S = Susceptible, PLR = Potential low-level resistance, LR = Low-level resistance, IR = Intermediate resistance, HR = High-level resistance)

Sample No.	Mutations detected		Resistance profile
	PR	RT	
DBS-008	None	D67Deletion, T69G, K70R, M184V, K219Q, K101H, K103N, G190A, Y318F	**S =** ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r **LR =** ETR, RPV **IR =** d4T, TDF **HR =** ABC, AZT, DDI, FTC, 3TC, EFV, NVP
DBS-010	None	M184V, Y181C	**S =** ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, NFV, SQV/r, TPV/r, AZT, d4T, TDF **PLR =** DDI **LR =** ABC **IR =** EFV, ETR, RPV **HR =** FTC, 3TC, NVP

(ATV/r, DRV/r, FPV/r, IDV/r, LPV/r, SQV/r, TPV/r = ritonavir boosted Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Saquinavir and Tipranavir respectively, NFV = Nelfinavir, ABC = Abacavir, AZT = Zidovudine, d4T = Stavudine, DDI = Didanosine, FTC = Emtricitabine, 3TC = Lamivudine, TDF = Tenofovir, EFV = Efavirenz, ETR = Etravirine, NVP = Nevirapine, RPV = Rilpivirine)

The mean number of mutations detected in plasma was 5.885. The agreement between plasma and DBS, and plasma and PBMC were 0.742 and 0.703, respectively.

When the sequences from the 20 samples which amplified in DBS were compared with corresponding PBMC sequences, 12 (60%) were found to be concordant. Among them, 8 were found to have identical drug resistance mutations and 3 did not have any drug resistance mutations. Although the remaining one sample had concordant resistance profiles, identified

mutations were slightly different between the two compartments. The details and summary of mutations detected in the concordant samples are listed in **Tables 39** and **40**.

Table 39: Sample with no drug resistant mutations detected in DBS and PBMC

Sample No.	Viral load in log_{10} copies/ml	Duration of treatment	Latest ART regimen	Treatment switches
DBS-003	4.52	3 years	Zidovudine + Lamivudine + Efavirenz	1 switch

(Samples DBS-013 and DBS-033 also did not have any drug resistance mutations in plasma, DBS and PBMC. The treatment details of these two individuals are already listed in **Table 27**).

The 8 samples with identical mutations in DBS and PBMC were DBS-001, DBS-006, DBS-016, DBS-030 and DBS-032 which are listed in **Table 28**, DBS-008 and DBS-010 which are listed in **Table 38** and DBS-015 which is listed in **Table 35** . None of these samples had mutations conferring resistance to protease inhibitors. All 8 had mutations that conferred resistance to non-nucleoside reverse transcriptase inhibitors and all except one (DBS-016) had mutations against nucleotide reverse transcriptase inhibitors.

The one sample which had an additional mutation in DBS was DBS-017 (corresponding with Plasma DR 17/05) is listed in **Table 29**. Despite having an extra mutation, H221Y, there was no difference in the drug resistant profiles between DBS and PBMC, and was considered to be concordant.

Of the 20 samples from DBS, 8 were found to be discordant with PBMC. Among the discordant samples, 6 PBMC sequences of samples DBS-002, DBS-004, DBS-007, DBS-012, DBS-025 and DBS-026 were identical to the sequences in plasma and are listed in **Tables 35** and **36**. The remaining 2 discordant samples, DBS-009 and DBS-024 had identical mutations in DBS and plasma and are listed in **Tables 30** and **31**.

Out of the total 29 samples, 9 did not amplify in DBS but amplified in PBMC. Of these, 7 PBMC samples (DBS-005, DBS-011, DBS-014, DBS-018, DBS-021, DBS-022 and DBS-023) had identical drug resistance profiles as that of plasma and are listed in **Table 37**. One sample (DBS-020) was discordant with plasma and is listed in **Tables 30** and **31**. And the last sample which amplified only in PBMC, did not have any detectable drug resistance mutations.

In the 20 DBS samples which amplified and was sequenced, mutations against PI, NRTI and NNRTI were seen in 5%, 80% and 85% of the samples, respectively. Among all the PBMC samples, mutations against PI, NRTI and NNRTI were seen in 6.9%, 72.4% and 79.3% of the samples, respectively. And among the 26 plasma samples which amplified and were sequenced, mutations against PI, NRTI and NNRTI were seen in 15.4%, 80.8% and 84.6% of the samples, respectively.

The mean number of mutations detected in DBS and PBMC were 4.550 and 4.621, respectively. The difference between the means of plasma and DBS was not statistically significant, $p = 0.952$. The difference between the means of plasma and PBMC was also not statistically significant, $p = 0.107$.

And the difference between the means of DBS and PBMC was not statistically significant, $p = 0.103$. The agreement between DBS and PBMC was 0.816. The Bland-Altman plot showed good overall agreement between DBS and PBMC in relation to plasma, with most values within 2SD as shown in **Figure 25**.

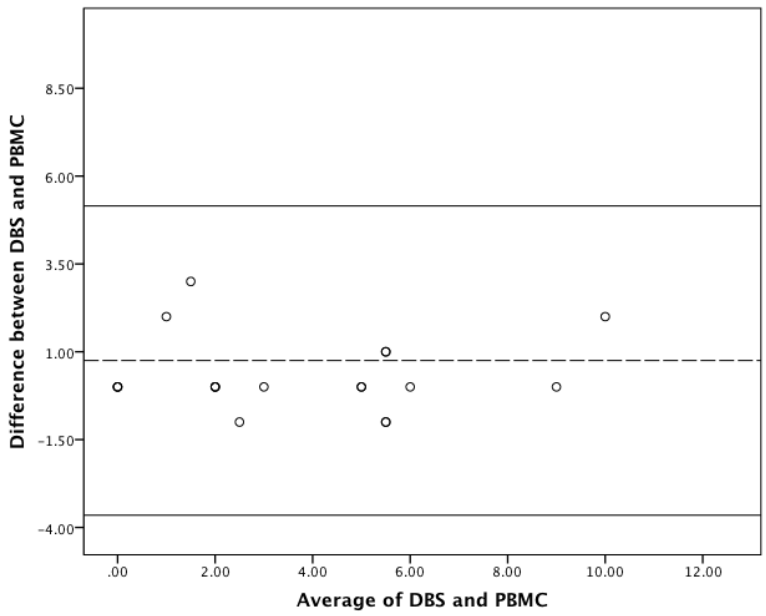


Figure 25: Bland-Altman plot showing overall good agreement

Next Generation Sequencing (NGS)

All three components of one sample was randomly selected for next genereation sequencing. The lowest frequency of a variant that was detected by NGS and not by conventional sequencing, from plasma, DBS and PBMC were 11.5%, 15.6% and 16.8% respectively. And the lowest frequency of a variant that was detected both by Sangers sequencing and NGS from plasma, DBS and PBMC were 33.3%, 32.4% and 23%, respectively. The impact of the detected minority variants on the drug resistance profile is yet to be studied.

6. Discussion

Human Immunodeficiency Virus (HIV) infection has developed into a global pandemic since its discovery in 1981, over 70 million people have been infected and about 35 million people have succumbed to it, worldwide. The end of 2016 had 36.7 million people living with HIV globally with an adult prevalence of 0.7-0.9% (24) and India had 2.117 million people living with HIV having an adult prevalence of 0.26% by 2015 (8,25). Of the two types of the virus, HIV-1 causes significantly more human infections worldwide, than HIV-2 (90). HIV is transmitted mainly through parenteral contact with infected body fluids and intravenous drug abuse, sexual contact and mother to child transmission (6). HIV predominantly targets CD4 T cells and progressively depletes it, leading to weakening of the immune system and results in Acquired Immunodeficiency Syndrome (AIDS) which is characterized by fatal opportunistic infections and malignancies (1,31).

Combination antiretroviral therapy (ART), is not a cure for HIV infection but it can suppress viral replication and alter the natural course of the disease from a progressive condition with a fatal outcome into a chronic manageable disease (31). As of year 2016, 19.5 million and 1.04 million people living with HIV had access to and were receiving ART in the world and India, respectively (7,9). Unfortunately, individuals who are on ART are under the perpetual threat of developing drug resistance, due to the high replication and mutation rate of the virus, the chronic nature of the infection and the necessity for lifelong treatment. Hence, arises the need for HIV drug resistance testing both for the management of HIV infected individuals and surveillance purposes (91).

Though plasma is the routinely used specimen for drug resistance genotypic testing, the need for early processing, sustaining cold-chain conditions and associated potential biohazard risks

has demanded alternate sample types in peripheral sites. With this background, dried blood spots (DBS) is being studied in resource limited settings as an alternative to plasma for HIV drug resistance testing, as it does not require immediate processing or temperature maintenance during transport and is considered non-infectious (11).

In our study conducted from July 2016 to August 2017, 29 individuals were included who were selected from among those who were suspected of treatment failure and referred for HIV-1 drug resistance testing and HIV-1 quantification. Among the recruited individuals, there were more men (79%) than women (21%) which was comparable with a study by Haile et al. where men were more likely to develop treatment failure than women with 83% of treatment failure being seen in men (92).

In our study, almost three-fourth of the participants belonged to age groups 31 - 40 (31%) and 41 – 50 years (41.4%) together with a median age of 46 years and an Interquartile range of 38 – 50 years and a study by Sang et al. showed a median age of 39 years with an Interquartile range of 32 – 48 years among individuals suspected of ART treatment failure (93).

Among our study participants, the median CD4 count at the time of drug resistance testing was 155 (Interquartile range: 60 – 325) cells/ μl and other studies like Sang et al. showed it to be 170 (Interquartile range: 96 - 277) cells/ μl (93) and Banks et al. had 148 (Interquartile range: 3 - 459) cells/ μl (94).

Banks et al. also showed a viral load log median of 4.95 copies/ ml with an Interquartile range of $\log_{10}$ 2.58 – 5.50 copies/ ml (94). While our study showed, median viral load among the individuals included in the study to be $\log_{10}$ 4.77 (Interquartile range: 3.74 – 5.34) copies/ ml.

Ayalew et al. found that most of the treatment failure cases were on a regimen of Lamivudine – Tenofovir backbone and poor adherence to ART was the strongest risk factor for treatment

failure. Non-adherent individuals were > 70 times more likely than individuals with good adherence to develop treatment failure (95). While Haile et al. found that among those in treatment failure, 43% were on Stavudine – Lamivudine – Nevirapine and 17% on Zidovudine – Lamivudine –Nevirapine while only 6% were on Zidovudine – Lamivudine – Efavirenz. However, these values are not a true representation of the prevalence of treatment failure, which would depend on the total number of persons taking each of these combination ARVs. The highest prevalence of treatment failure was noted in individuals taking Zidovudine – Lamivudine – Efavirenz and the lowest was among individuals on Tenofovir – Lamivudine – Efavirenz (92). In our study, 13 different combination ART regimens were followed by different participants and almost all were on 3 drug combination therapies. About 39% of the individuals were on Tenofovir – Lamivudine/ Emtricitabine – Efavirenz regimen and roughly 28% had poor adherence to the regimen they were on. Though the remaining individuals had good adherence, they had detectable levels of viral load and drug resistance mutations, probably due to following regimens with drugs with low genetic barrier to drug resistance combined with drug pressure.

Keiser et al. showed 2.9% switch from first-line to second-line ART regimen among their study population (96). Similarly, NACO states that there has been 2.3% switch to second line regimen and 0.4% switch to third-line treatment regimen among the individuals on ART in India (70). Among our study participants, 14% and 3.6% had switched to second and third-line treatment regimen, respectively. The higher rates of treatment switch seen in our study is probably due to the smaller sample size and inclusion of only individuals suspected of treatment failure.

Dragsted et al. found that most instances of treatment failure took place early after ART initiation. A decreasing trend was seen in the rate of failure with each additional year (97). Treatment failure within the early months of ART initiation may be due to poor adherence, drug toxicity leading to ART discontinuation, selection of resistant strains or suboptimal regimens, as listed by Netsanet et al. In our study, 32% of the participants developed treatment failure within 24 months of starting ART and only 14% after 10 years of therapy.

Devi SB et al., in their study found that tuberculosis (pulmonary, extra-pulmonary and disseminated) and candidiasis (all sites) were the most prevalent opportunistic infections seen among people on ART making up 25.4% and 16.6% of the cases, respectively. This was followed by bacterial infections, cryptococcosis and *P.jiroveci* infection. And in individuals with treatment failure, the commonly seen opportunistic infections were tuberculosis, oral candidiasis, gastrointestinal infection with *Cryptosporidium* and *Isospora*, cryptococcosis and *P.jiroveci* infection (98). Another study by Sachithanandham at al. specifically looked for opportunistic viral infections in treatment-naïve HIV infected individuals in South India from whole blood samples and found 72% of EBV and 7% of CMV infections (99). Among our study participants suspected of treatment failure the frequency of the commonest opportunistic infections were as follows, tuberculosis was seen in 52%, candidiasis in 24%, herpes zoster in 17%, cutaneous fungal infections, CMV retinitis and cryptococcal meningitis was seen in 7% of individuals. Sachithanandham at al. did not find any individual infected with HSV-1, HSV-2, VZV and JC virus, probably because whole blood was the sample tested, our study participants did however have herpes zoster, herpes genitalis and progressive multifocal leukoencephalopathy.

Most of the available data on HIV-1 drug resistance to ART are for subtype B HIV-1, which is prevalent in developed countries but is responsible for only about 12% of global infections. While non-B subtypes account for roughly 88% of all infections and are seen mainly in low and middle-income countries (100). In our study, as expected all the infections were caused by HIV-1 subtype C which is the predominant subtype seen in India.

Our study, compared DBS stored at 25^0-30^0C to plasma for the detection of HIV-1 drug resistance among treatment-experienced individuals using in-house amplification assays.

Proper RNA extraction is necessary for good recovery of HIV-1 RNA in order to ensure the success of downstream molecular analysis. Automated NucliSENS easyMag extraction system is used by most reference laboratories for HIV RNA extraction. Although it is highly efficient, it requires specialized instruments and accessories which are usually unavailable and expensive in resource-limited scenarios. Gupta et al. assessed the QIAamp Viral RNA spin column-based manual extraction and compared it to automated NucliSENS easyMag extraction and found it to be equally competent, more practical, economical and more easily available. They also studied the optimal plasma input volume at different viral load levels and found that an initial plasma input volume of 400 µl ensures downstream success in samples with low viral load. In our study, we used QIAamp viral RNA extraction kit for HIV-1 RNA extraction from plasma, as per the manufacturer's instructions. And in unamplified low viral load samples, we modified the manufacturer's protocol and used a plasma input volume of 400 µl instead of the recommended 140 µl (101).

Successful amplification and sequencing of HIV-1 RNA from plasma depends on the viral load in a sample (102). In a study done by Diallo at al. they found an amplification success rate of 87% from plasma by using a broadly sensitive in-house assay (91). Zhou et al. studied

non-B subtypes detected from treatment experienced individuals' plasma with viral load ≥ and < 3 $\log_{10}$ copies/ ml and obtained 100% and 78.6% success rates, respectively (103). Among our plasma samples, 96.2% (25/26) amplified when the viral load was ≥ 3 $\log_{10}$ copies/ ml which was comparable with the findings of other studies. One sample with VL = $\log_{10}$ 3.4 copies/ ml which did not amplify belonged to an individual who was on ART for 15 years and had been switched to second-line therapy 7 months prior to testing. And when the VL was < 3 $\log_{10}$ copies/ ml, only one-third of the samples amplified, which was less than Zhou et al. where more than three-fourth of samples had amplified.

Zhou et al. like most studies done on Dried Blood Spots (DBS) used NucliSens easyMAG automatic system to extract blood total nucleic acid (TNA) using 1 spot cut out from the filter paper card and followed the manufacturer's protocol (103). For extraction of nucleic acid from DBS we took 3 punches of the spot and used QIAamp DNA Mini extraction kit which co-purifies both DNA and RNA. Then performed separate amplification assays, direct nested PCR and RT-PCR followed by nested PCR for DNA and RNA amplification, respectively. Performing separate amplification for DNA and RNA from each sample is probably more cumbersome yet it is less expensive and more easily available in most laboratories than the automated extraction instruments.

Several studies have been conducted to evaluate the use of DBS for HIV-1 genotyping and have had varying success. *pol* gene amplification has been done using diverse testing methods. The reported amplification sensitivity from DBS markedly differs between assays with some in-house tests (Buckton et al.(104)) having successful amplification from DBS at low viral loads. All reports indicate slightly lower amplification sensitivity of DBS than plasma. Genotypic sensitivity of 1000 copies/ml was attained by only two laboratories in a

recent WHO proficiency testing (105). Our study in which the DBS cards were stored at 25^0-30^0C for 10 days and then at - 20^0C showed 73% amplification success in samples with VL > 1000 copies/ ml. While Parry CM et al. had around 90% and 63% success rate in DBS cards kept at ambient temperature for 2 weeks and 4 weeks, respectively, when the viral load ranged from 1000-10000 copies/ ml (105).

Our study had 5 samples with high viral load ranging from $\log_{10}$ 4.98 – 5.79 copies/ ml which did not amplify in DBS, despite repeat testing. These cards were examined to confirm the integrity of the filter paper card and was found to be the same as the other card samples. The presence of mutations at the primer binding site was considered, however, since the corresponding plasma and PBMC samples amplified, it was unlikely. To rule out higher concentration of nucleic acid, fewer punches were taken, the extract was diluted and retested. New batch of reagents were used for the repeat testing. All the packed DBS cards were stored in in the same container at - 20^0C until testing but the storage time varied between samples. Probably prolonged storage at - 20^0C adversely affected the outcome of these 5 DBS cards.

While the lowest viral load that amplified from DBS was $\log_{10}$ 2.88 copies/ ml. The efficiency of HIV-1 genotyping from DBS depends on the methodology of the assay and storage conditions, specifically, humidity and temperature (105).

The mean number of mutations detected in plasma, DBS and PBMC were 5.885, 4.550 and 4.621, respectively in our study. The difference between the means of plasma, DBS and PBMC were not statistically significant. Plasma samples may have a higher mean than PBMC samples, since only archived mutations will be detected from PBMC. Whereas, DBS samples are expected to have a higher number of mutations than plasma, however, in our study it was lower, since the number of DBS samples that amplified was lesser than plasma.

Drug mutations detected in the study by Zhou et al. showed 90.4% concordance between plasma and DBS, 7.5% were partially discordant and only 2.1% were completely discordant (103). On comparing drug resistance mutations between 18 matched samples which amplified both in plasma and DBS from our study, 10 had identical resistance profiles, 5 samples had no significant change in resistance profile and only 3 (16.7%) samples were completely discordant. Different factors could lead to these differences seen between DBS and plasma sequences like: proviral DNA in DBS having mutations different from those seen in free virus, inconsistent amplification of viral quasispecies or variants, primer binding preference, sequence quality, base-calling variability (103,105).

DBS is an appropriate sample type for HIV drug resistance surveillance in resource-limited settings if it is carefully prepared and stored at ambient temperatures for 2 weeks or less (105).

In addition to plasma and DBS, drug resistance mutations can be detected from peripheral blood mononuclear cells (PBMC) which may be easier and less expensive. The archived mutations will be retained in proviral DNA, despite the absence of drug pressure due to discontinuation or alteration in the regimen but there are still unanswered questions regarding its clinical significance (94).

Diallo et al. had a success rate of 97% from PBMC which was extracted using QIAamp DNA kit and amplified directly using an in-house nested PCR (91). In our study, irrespective of the viral load levels all samples amplified giving a 100% amplification success rate from PBMC. The fragile and easily degradable nature of RNA becomes less than ideal for HIV drug resistance testing, when compared to DNA molecule, particularly in unreliable cold chain conditions often seen in resource-limited settings. So, despite both plasma and PBMC

requiring cold chain conditions for storage and transportation, PBMC DNA could be an alternative for drug resistance testing in resource-limited countries. Additionally, genotyping proviral DNA from PBMC could be convenient in individuals with VL < 3 $\log_{10}$ copies/ ml (91).

Derache et al. through their study, showed 84% concordance between proviral DNA and plasma RNA drug resistance profiles among viremic study subjects (106), to which our study was similar. On comparing drug resistance mutations between 26 matched samples which amplified both in plasma and PBMC from our study, 21 had identical resistance profiles, 1 sample had no significant change in resistance profile and 4 (15.4%) samples were completely discordant. Also out of the 3 samples which did not amplify in plasma but amplified in PBMC, 2 samples had mutations which rendered them resistant to 2 classes of ARVs (NRTI and NNRTI).

It was noticed that one sample which had 11 mutations in plasma, did not have any detected mutations from the corresponding DBS and PBMC samples. To rule out the possibility of contamination, the plasma sample was retested and was found to have the same mutations again. The sequences obtained from DBS and PBMC were analysed again to look for any mutations that may have been missed. Since DBS represents whole blood, the mutations seen in plasma RNA is expected to be seen here as well, even if it is absent from PBMC. Also it is unlikely that all 11 mutations were new and had not been archived into proviral DNA. The complete lack of mutations in both DBS and plasma, while plasma had multiple mutations was unexpected and need further analysis.

Another 2 samples were noticed to have 3 and 2 mutations in DBS, while the corresponding plasma and PBMC samples did not have any mutations. The plasma samples were repeat

tested and were found to be free of mutations again. One of the DBS samples which was retested, showed the presence of drug resistant mutations again. The mutations detected in a DBS sample are either from the free viral RNA or the proviral DNA. Hence, if mutations are detected from a DBS sample, they must also be present in plasma or PBMC, if not both. Here again, the lack of mutations in plasma and PBMC, while mutations being detected in the corresponding DBS samples was unexpected and warrant further analysis.

The frequency of mutations detected against NRTI and NNRTI were similar in the sequenced plasma, DBS and PBMC samples. However, the frequency of mutations against PI in plasma, DBS and PBMC were 15.4%, 5% and 6.9%, respectively. This difference between plasma and DBS was due to 3 samples with PI mutations which did not amplify in DBS. And the difference between plasma and PBMC was probably due to newer mutations not being detected in PBMC.

Genotyping of proviral DNA from PBMC may lead to detection of drug resistance which may be complimentary to plasma RNA mutations. Archived mutations in proviral DNA denotes resistance which is maintained in the absence of therapy whereas resistance mutations in plasma relies on adherence and treatment exposure. For sustainable surveillance purposes, using whole blood might be more feasible and proviral DNA sequences to trace the prevalence of resistance mutations (94).

Conventional sequencing, merely detects variants with a frequency $\geq$ 20% in an infected individual. Whereas, next generation sequencing (NGS) can detect minority variants at very low frequencies. This may help to recognise the actual rate of drug resistant variants in treatment-naïve and -experienced persons (68). In our study, NGS done on the 3 components

of one sample, detected mutant variants at frequencies < 20% but their influence on the drug resistance profile is yet to be looked into.

The main limitation of our study, was that the sample size was too small to clearly assess the genotypic testing efficacy between the different sample types.

The storage temperature was not as extreme as it might be in real-life scenarios since the cards were packed properly and left in an air-conditioned room. DBS was spotted with a fixed volume of venous blood, using pipettes in a laboratory setting which may not reflect the actual impact of practical weather conditions and suboptimal DBS preparation and storage by an inexperienced personnel in resource-limited settings. Proper packaging and storage of DBS are vital for successful genotyping (105).

This study may help start the possibility of a cost effective, DBS stored at room temperatures for HIV-1 drug resistance surveillance in resource-limited settings for the purpose of developing national policies and ART guidelines. The use of DBS will aid in the collection of country-wide representative samples by including specimens from rural locations which will be critical to the launch of a National programme.

7. Summary and Conclusion

Summary

1. This study was done to evaluate the efficacy of DBS stored at 25^0-30^0C when compared to plasma for the detection of HIV-1 drug resistance among treatment-experienced individuals infected with HIV-1 subtype C strain, using an in-house assay and to look for the degree of concordance between plasma and DBS mutations.
2. Additionally we looked at drug resistance mutations from PBMC and its concordance with mutations detected in plasma and DBS.
3. Blood sample from 29 treatment experienced individuals referred for drug resistance testing were collected, spotted onto DBS cards which were then stored at 25^0-30^0C for 10 days, and plasma and PBMC were separated and stored.
4. Each sample type was tested for HIV-1 drug resistance mutations by genotypic drug resistance testing which involved amplifying the *pol* gene using a nested PCR and then sequencing it.
5. From the 29 study subjects, 79% were men and the predominant (41.4%) age group was 41-50 years when men and women were taken together.
6. The median CD4 count among the participants was 155 (Interquartile range: 60 – 325) cells/ μl and 37.9% of them had CD4 cell count < 100 cells/ μl.
7. The median viral load expressed as $\log_{10}$ copies/ ml was 4.77 (Interquartile range: 3.74 – 5.34) $\log_{10}$ copies/ ml with almost 90% of study subjects having a viral load > 3 $\log_{10}$ copies/ ml.
8. Between the 28 participants whose ART regimen was known, there were 13 different combinations followed, 46.4% had never switched their treatment regimen, 4 were on

second-line ART using boosted Protease Inhibitors and 1 was on third-line ART regimen which included an Integrase Inhibitor as well.

9. The median duration of having received ART was 5 (Interquartile range: 1.13 - 10) years but 32.1% of participants had received ART for < 2 years.
10. Among the 26 samples with viral load > $\log_{10}$ 3 copies/ ml, 25 (96.2%), 19 (73.1%) and 26 (100%) samples amplified from plasma, DBS and PBMC, respectively.
11. Among the 3 samples with viral load < $\log_{10}$ 3 copies/ ml, 1 (33.3%), 1 (33.3%) and 3 (100%) samples amplified from plasma, DBS and PBMC, respectively.
12. Totally, 26 samples amplified in plasma out of the 29 included in the study and 8 of these did not amplify in DBS, of which 5 had high viral load levels.
13. Of the 20 samples which amplified in DBS out of the 29 included in the study, 2 did not amplify in plasma of which one had viral load > 3 $\log_{10}$ copies/ ml.
14. Of the total 29 samples, between plasma, DBS and PBMC, 18 samples amplified in all three sample types. Ten samples were sucessfully amplified in any 2 sample types and one sample amplified only from one sample type which was PBMC. Thus PBMC showed the highest (100%) amplification sensitivity among the 3 sample types.
15. The overall sensitivity of DBS and PBMC with respect to plasma was 69.2% and 100%, respectively.
16. The mean number of mutations in plasma, DBS and PBMC were 5.885, 4.550 and 4.621, respectively. The difference between the means were not statistically significant.
17. The agreement between plasma and DBS, and plasma and PBMC were 0.742 and 0.703, respectively. And the agreement between DBS and PBMC was 0.816.
18. Also out of the 3 samples which did not amplify in plasma but amplified in PBMC, 2 samples had mutations which rendered them resistant to NRTIs and NNRTIs.

19. In the 20 DBS samples which amplified and were sequenced, mutations against PI, NRTI and NNRTI were seen in 5%, 80% and 85% of the samples, respectively.
20. Among all the PBMC samples, PI, NRTI and NNRTI mutations were seen in 6.9%, 72.4% and 79.3% of the samples, respectively.
21. Among the 26 plasma samples which amplified and were sequenced, mutations against PI, NRTI and NNRTI were seen in 15.4%, 80.8% and 84.6% of the samples, respectively.
22. The lowest frequency of a variant that was detected by NGS from plasma, DBS and PBMC were 11.5%, 15.6% and 16.8% respectively.

Limitations

The sample size was too small to clearly assess the genotypic testing efficacy between the different sample types. And inexplicably 5 samples with high viral load which amplified in plasma and PBMC did not amplify from DBS, despite repeat testing, troubleshooting and modifying the protocol. Also, in our study reproducibility was studied only minimally, whereas it needs to be looked into extensively.

Conclusion

In conclusion, DBS stored at 25^0-30^0C for 10 days had 73.1% success rate for HIV-1 genotyping in samples with > 3 $\log_{10}$ copies/ ml. Thus DBS is a promising sample for HIV-1 genotyping in resource limited settings due to ease of collection, storage and transportation. Additionally PBMC showed 100% amplification and good correlation with plasma. Though the impact of archived mutations is still unclear for individuals failing their current regimen, it can be considered complimentary to plasma for drug resistance surveillance and as an alternative in cases of low viral load levels.

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Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

June 16, 2016

Dr. Priyanka Sabu,
PG Registrar,
Department of Clinical Microbiology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research Funding: New Proposal**
Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations.
Dr. Priyanka Sabu (Employment Number: 21198), Post graduate Registrar, Department of Clinical Microbiology, Dr. Rajesh Kannangai, Employment Number: 20093, Clinical Virology, Dr. John G Fletcher, Clinical Virology, Dr. S. Jaiprasath, Clinical Virology, Mr. John Paul Demosthenes, Clinical Virology, Mrs. R. Veena Vadhini, Clinical Virology, Dr. D.R. Naveen Kumar (Employment no: 33632),, Clinical Microbiology, Dr. Visalakshi Jeyaseelan (Employment no: 31093), , Biostatistics.,

Ref: IRB Min No: 9832 [DIAGNO] dated 07.01.2016

Dear Dr. Priyanka Sabu,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
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SECRETARY - (ETHICS COMMITTEE)
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11/8/16

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June 16, 2016

Dr. Priyanka Sabu,
PG Registrar,
Department of Clinical Microbiology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research Funding: New Proposal**
Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations.
Dr. Priyanka Sabu (Employment Number: 21198), Post graduate Registrar, Department of Clinical Microbiology, Dr. Rajesh Kannangai, Employment Number: 20093, Clinical Virology, Dr. John G Fletcher, Clinical Virology, Dr. S. Jaiprasath, Clinical Virology, Mr. John Paul Demosthenes, Clinical Virology, Mrs. R. Veena Vadhini, Clinical Virology, Dr. D.R. Naveen Kumar (Employment no: 33632),, Clinical Microbiology, Dr. Visalakshi Jeyaseelan (Employment no: 31093), , Biostatistics.,

Ref: IRB Min No: 9832 [DIAGNO] dated 07.01.2016

Dear Dr. Priyanka Sabu,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations" on January 07th 2016.

The Committee reviewed the following documents

1. IRB Application format
2. Patient Information Sheet and Informed Consent Form (English, Tamil, Hindi, Telugu)
3. Proforma
4. Cvs of Drs. . Priyanka Sabu, Rajesh Kannangai, Jaiprasath, . Naveen Kumar, Visalakshi Jeyaseelan, Mrs. R. Veena Vadhini, Mr. John Paul Demosthenes
5. No. of documents 1- 4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on January 07th 2016 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician
Dr. RV. Shaji		Professor, Heamatology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. B. J. Prashantham	MA(Counseling Psychol MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centr Vellore	External, Social Scientist
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Anand Zachariah	MBBS, PhD	Professor, Medicine, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MD, DNB(Endo), Phd(Endo)	Professor, Endocrinology, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Fluid Grant Allocation:
A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for22Months. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 9832 [DIAGNO] dated 07.01.2016

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD
CHRISTIANMEDICALCOLLEGE,
BAGAYAM, VELLORE 632002, TAMIL NADU, INDIA

Ref: FG/9832/01/2016

August 12, 2016

Mr. Robby Pria Sundersingh
The Treasurer
Christian Medical College,
Vellore.

Dear Mr. Robby Pria Sundersingh,

Sub: **Fluid Research Funding: New Proposal**
Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations.
Dr. Priyanka Sabu (Employment Number: 21198), Post graduate Registrar, Department of Clinical Microbiology, Dr. Rajesh Kannangai, Employment Number: 20093, Clinical Virology, Dr. John G Fletcher, Clinical Virology, Dr. S. Jaiprasath, Clinical Virology, Mr. John Paul Demosthenes, Clinical Virology, Mrs. R. Veena Vadhini, Clinical Virology, Dr. D.R. Naveen Kumar (Employment no: 33632),, Clinical Microbiology, Dr. Visalakshi Jeyaseelan (Employment no: 31093), , Biostatistics

Ref: IRB Min. No. 9832 dated 07.01.2016

The Institutional Review Board at its meeting held on January 07th 2016 vide IRB Min. No. 9832.Accepted the project for *A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/Annual report and subsequent submission of it to the IRB.*

Kindly arrange to transfer the sanctioned amount to a separate account to be operated by Dr. Priyanka Sabu (priyankasabu.ps@gmail.com) and Dr. Rajesh Kannangai(kannangair@cmcvellore.ac.in)

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board, CMC, Vellore.

Dr. BIJU GEORGE
MBBS, MD, DM
SECRETARY (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

CC: Dr. Priyanka Sabu, Department of Clinical Microbiology, CMC, Vellore
Dr Rajesh Kannangai, Department of Clinical Virology, CMC, Vellore
File

Informed Consent Form

Informed Consent form to participate in a research study

Study Title: Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations.

Study Number: _____

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age: _____

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that investigator, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s). [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable

Date: _____/_____/_____

Signatory's Name: _____ Signature:

Or

Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature (or) thumb impression of the Witness: _____

Date: _____/_____/_____

Name and Address of the Witness: _____

PATIENT INFORMATION SHEET

Date: ____/____/_____

Study title: Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations.

Please read this sheet carefully. It gives you important information about this study. If you have any questions about the study, please ask us. If you decide to take part in this study, please sign or provide a thumb impression on provided consent form to show that you are willing to take part.

Why is this study being done?

HIV infection is an important global health issue and causes severe morbidity and mortality. Individuals who are on treatment for HIV infection, but not improving are tested for drug resistance. Detection of drug resistance will help in deciding the changes to be made in the treatment regimen. Routinely your blood is collected in a sample collecting tube for performing these virological tests.

Due to lack of good laboratory services in remote parts of our country, many infected individuals are not monitored adequately. Blood samples in test tubes cannot be easily sent to distant laboratories. From the blood sample drawn from you as part of your routine management, about 0.5ml of your blood will be spotted onto a filter paper and then used for detection of HIV-1 drug resistance. The efficacy of these dried blood spots to detect drug resistance will be compared with the routine method. If shown to be equally effective, it can be used in resource limited settings, as a means of sample collection, storage and transport to distant referral laboratories.

What will happen in this study?

In this study, a fraction (0.5ml) of blood obtained from you as part of routine testing will be spotted on a filter paper and then used to check the efficacy of dried blood spots as a method of sample collection, storage and transportation.

Will I be paid to take part in this study?

No study participant will be paid.

What are the risks and possible discomforts from being in this study?

There is no additional risk by participating in this study.

What are the possible benefits from being in this study?

You will not benefit personally from taking part in this study. However, your participation will help us evaluate dried blood spots as an alternate method for sample collection, storage and transport. This might benefit patients in resource limited settings in the future.

Will I be told of the results of the study?

The results obtained from plasma will be revealed to the study subjects but the results of DBS sample will be used only for the study purposes.

If I refuse to take part in this study, will it affect my healthcare?

You are free to refuse to participate in this study. Should you do so, it will not affect your treatment in this institution in any way.

If I take part in this research study, how will you protect my privacy?

Information collected from you for this study will be available only to the investigators. Study participants will be identified only by a unique ID number. Your name and other information, will be kept confidential and your blood sample will be identified by your unique ID number. The study consent form you sign may be inspected by regulatory agencies or the Institutional Review Board in the course of carrying their duties.

If I have questions or concerns about this research study, whom should I call?

You can call me if you have further questions or concerns. Details are as below:

Dr. Priyanka Sabu

PG Registrar

Department of Clinical Microbiology

Christian Medical College,

Vellore - 632 004,

Phone: +919591257164

PATIENT PROFORMA

Study Title: Evaluation of efficacy of Dried Blood Spots (DBS) as compared to plasma samples for the estimation of HIV-1 drug resistance mutations.

Study number: **Date:**

Name:

Hospital number:

Date of birth/ Age/Sex:

Address:

State:

Mother tongue:

Marital status:

Occupation:

Date of HIV diagnosis:

Date of starting ART:

ART regimen followed:

Clinical diagnosis: WHO stage I/II/III/IV

Mode of diagnosis:

History of chronic diarrhoea

Weight loss

Fever

Cough

History/ Diagnosed OI/ Other infections: (This information will be obtained retrospectively from the clinical workstation/ patient work chart)

a) Tuberculosis, b) Candidiasis, c) Herpes Zoster, d) Pneumocystis carinii pneumonia, e) Bacterial pneumonia, f) Salmonellosis, g) Toxoplasmosis, h) Cryptosporidiosis, i) CMV, j) Cutaneous fungal infections, k) Pelvic inflammatory disease, l) Cryptococcal meningitis, m) Histoplasmosis, n) Hepatits

Follow up Date:

CD4+/ CD8+ T cell count:

Viral load:

Sample collected by:

Name: Signature:

Data Sheet

Plasma ID	Sample ID	Age	Sex	State	CD4	Viral load	Duration	ART	Opportunistic Infections	Staging	Diagnosed on
DR 16/29	DBS-001	36	M	TN	37	57,465	9 months	TDF/3TC/EFV	Herpes zoster	II	2012
DR 16/31	DBS-002	40	M	AP	357	2,66,164	3.5 years	NVP/d4T/3TC > NVP/TDF/3TC > TDF/3TC/EFV	TB lymphadenitis	IV	20I2
DR 16/33	DBS-003	35	M	Jharkhand	16	33,231	3 years	AZT/3TC/NVP > AZT/3TC/EFV	DisseminatedTB, Candidiasis	IV	Dec-12
DR 16/34	DBS-004	48	F	TN	414	8,842	12 years	d4T/3TC * 4 years	Nil	I	2002
DR 16/35	DBS-005	51	M	Kerala	75	6,17,560	1 year	TDF/FTC/EFV	Pulmonary TB	III	Jun-15
DR 16/36	DBS-006	47	F	TN	78	17,35,708	1 year	AZT > TDF/FTC/EFV	Oral candidiasis, CMV retinitis, Cutaneous mycosis	IV	Apr-15
DR 16/37	DBS-007	43	M	AP	228	14,066	1 year	TDF/FTC/EFV	Disseminated TB	IV	Jun-15
DR 16/38	DBS-008	18	M	AP	309	2,484	15 years	TDF/FTC/EFV > Feb 2016 TDF/FTC/AV/r	TB, HIV asso. Neurocognitive disorder, EBV in CSF	IV	2001
DR 16/41	DBS-009	54	M	AP	289	1,037	6 years	d4T/3TC/EFV> d4T/3TC/NVP> TDF/FTC/LPV/r> TDF/FTC/DRV/r> AZT/3TC/RAL/DRV/r	Pulmonary TB	IV	1998
DR 16/42	DBS-010	39	F	TN	138	754	7 years	d4T/3TC/NVP > discontinued > Jan 2016 d4T/3TC/NVP	PCP	IV	2009
DR 16/43	DBS-011	35	F	AP	330	2,381	5 years	2011 TDF/3TC/EFV > discontinued > Jan 2013 AZT/3TC/NVP	Nil	I	2011
DR 16/44	DBS-012	45	F	AP	348	58,242	10 years	TDF/FTC/EFV > ABC/3TC/EFV	Progressive Multifocal Leucoencephalopathy	IV	May-06
DR 16/45	DBS-013	48	M	AP	155	16,50,289	10 years	2011 TDF/FTC/EFV > discontinued > Feb 2014 restarted	TB	IV	2006
DR 17/01	DBS-014	52	M	TN	11	96,180	16 years	1997 AZT/DDI/IDV/SQV 2005> Lost> 2013 TDF/FTC/EFV> discontinued > 2015 restarted	TB, Herpes zoster, Hepatitis C Chr.diarhea, Cognitive decline	IV	1997
DR 17/02	DBS-015	46	M	AP	308	1,26,171	5 years	AZT/3TC/NVP	Herpes zoster	II	2009
DR 17/03	DBS-016	45	M	AP	579	54,111	2 years	AZT/3TC/NVP > discontinued NVP > March 2015 TDF/3TC/NVP	Nil	I	Aug-14
DR 17/05	DBS-017	56	M	Bihar	13	32,332	1.5 years	TDF/3TC/EFV	Disseminated TB	IV	Jun-15
DR 17/07	DBS-018	55	M	TN	76	1,38,018	8 years	AZT3TC/EFV - irregular	Herpes zoster	II	2008
DR 17/08	DBS-019	36	M	TN	184	615	9 years	AZT/3TC/NVP	PCP	IV	2009
DR 17/09	DBS-020	47	M	Kerala	775	881	11 years	Feb 2006 AZT/3TC/NVP > 2011 TDF/3TC/ATV/r > TDF/3TC/LPV/r	Pulmonary TB	III	1999
DR 17/12	DBS-021	33	M	AP	5	2,45,303	4 years	AZT/3TC/NVP > TDF/3TC/NVP > TDF/3TC/EFV	Oral candidiasis, PCP, Cryptococcal meningitis	IV	Jin-2013
DR 17/13	DBS-022	37	M	AP	107	1,88,253	10 years	2010 Stopped 2 yrs > 2012 TDF/FTC/EFV > Jan 2017 TDF/3TC/ATV/r	TB, oral candidiasis, neuro-cognitive disorder	IV	2007
DR 17/15	DBS-023	39	M	TN	159	3,382	10 years	2008 AZT/3TC/NVP > 2013 TDF/3TC/EFV > 2016 TDF/FTC/ATV/r	CMV retinitis, Isosporiasis	IV	2007
DR 17/16	DBS-024	44	F	TN	52	34,791	3 months	d4T/3TC/EFV	Disseminated TB, oral candidiasis	IV	2014
DR 17/17	DBS-025	54	M	TN	319	1,85,107	9 years	d4T/3TC/NVP	Herpes zoster	II	2008
DR 17/18	DBS-026	49	M	TN	44	8,41,687	1 year	TDF/FTC/EFV	TB, oral candidiasis, Herpes genitalis	IV	Jun-16
DR 17/20	DBS-030	47	M	TN	479	1,83,878	3 years	2014 Irregular > 2016 ABC/3TC/EFV	TB	III	1998
DR 17/21	DBS-032	56	M	TN	108	6,05,687	1 year	ABC/3TC/NVP > TDF/3TC/EFV	Disseminated TB	IV	May-16
VL 17/375	DBS-033	47	M	AP	68	1,95,236	Not known	Not known	Oral candidiasis, Cryptococcemia + meningitis, Cutaneous mycosis	IV	2005

M = Male F=Female

TN = Tamil Nadu

AP = Andhra Pradesh

ATV/r, DRV/r, IDV/r, LPV/r, SQV/r = ritonavir boosted Atazanavir, Darunavir, Indinavir, Lopinavir and Saquinavir, respectively,

ABC = Abacavir, AZT = Zidovudine, d4T = Stavudine, DDI = Didanosine, FTC = Emtricitabine, 3TC = Lamivudine, TDF = Tenofovir, EFV = Efavirenz, NVP = Nevirapine,

RAL = Raltegravir